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(54) Title: CATIONIC VIROSOMES AS TRANSFER SYSTEM FOR GENETIC MATERIAL

(57) Abstract

The present invention relates to a positively charged virosome for efficient delivery of genetic material to resting or proliferating mammalian cells in vitro and in vivo. The virosome membrane contains cationic and/or polycationic lipids, at least one viral fusion peptide and preferably at least one cell-specific marker, advantageously selected from the group consisting of monoclonal antibodies, antibody fragments $F(ab')_2$ and Fab', cytokines, and growth factors, for a selective detection and binding of target cells. The invention further relates to a method for the manufacture of the novel virosomes and to applications thereof, particularly for the manufacture of pharmaceutical compositions to treat cancer or leukemia.

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CATIONIC VIROSOMES AS TRANSFER SYSTEM FOR GENETIC MATERIAL

FIELD OF INVENTION

The present invention is in the field of gene biotechnology and gene therapy and relates to novel virosomes, i.e., positively charged liposomal vesicles containing viral glycoproteins in the membrane, for efficient transfer of genetic material to target locations, a method of manufacture and useful applications thereof. The present cationic virosomes are particularly suitable for the specific and unspecific, non-infectious delivery of genes to target cells *in vitro* and *in vivo*.

BACKGROUND OF THE INVENTION

Liposomes are widely used as carriers for drug delivery and as protective shelters for short-lived pharmaceutical substances or against (bio)chemical attack by bodily fluids. Liposomes containing reconstituted membrane proteins or parts thereof from viral envelopes are usually called "virosomes" (Sizer et al., Biochemistry 26:5106-5113, 1987). They have been applied for non-specific delivery of various drugs and DNA molecules (Vainstein et al., Methods Enzymol. 101:492-512, 1983). It turned out to be a major drawback that these virosomes fused with the cell membrane of the target cells resulting in an uncontrolled release of the transported material into the cytoplasm of the target cells where the unprotected material was readily attacked by degradative intracellular processes.

WO 92/13525, the whole contents of which shall herewith be incorporated by reference, reports that virosomes made of phospholipid bilayer membranes which are targeted with viral spike proteins from influenza virus and with cell-specific markers such as, e.g., monoclonal antibodies, very efficiently fuse with model membranes and animal cells due to a virus-like penetration mechanism by way of receptor-mediated endocytosis. While these virosomes are successfully applied to deliver chemical substances and desired drugs to target locations, they suffer from certain disadvantages with respect to stable incorporation and transfer of charged molecules such as, for instance, negatively charged nucleic acids.

Within the last few years the delivery, notably the cell-specific delivery, of genetic material incorporated in liposomes has gained more and more

attention and importance, particularly with regard to applications in anti-cancer and gene therapy. Several methods are currently available for delivery of DNA or RNA to cells: Virus mediated methods, lipid mediated methods, and other methods like microinjection and electroporation. The advantages and disadvantages of current gene transfer techniques can be summarized as follows:

a) Virus mediated gene transfer: Genes can be introduced stably and efficiently into mammalian cells by means of retroviral vectors. However, the efficiency of gene transfer to non-replicating cells is very low because retroviruses infect only dividing cells. Further, general safety concerns are associated with the use of retroviral vectors relating to, for instance, the possible activation of oncogenes. Replication-defective adenovirus has become the gene transfer vector-of-choice for a majority of investigators. The adenovirus vector mediated gene delivery involves either the insertion of the desired gene into deleted adenovirus particles or the formation of a complex between the DNA to be inserted and the viral coat of adenovirus by a transferrin-polylysine bridge. The drawback of this very efficient system *in vivo* is an undefined risk of infection or inflammation: Despite the E1 gene deletion that renders the virus defective for replication, the remaining virus genome contains numerous open reading frames encoding viral proteins (Yang et al. 1994; Proc. Natl. Acad. Sci. USA 91, 4407-4411). Expression of viral proteins by transduced cells elicits both humoral and cellular immune responses in the animal and human body and thus, may provoke inflammation and proliferation.

In the HVJ (Sendai virus) mediated method the foreign DNA is complexed with liposomes. The liposomes are then loaded with inactivated Sendai virus (hemagglutinating virus of Japan; HVJ). This method has already been used for gene transfer *in vivo* to various tissues. In addition, cellular uptake of antisense oligonucleotides by HVJ-liposomes was reported (Morishita et al. 1993; J. Cell. Biochem. 17E, 239). A particular disadvantage is, however, that the HVJ-liposomes tend to non-specifically bind to red blood cells.

b) Lipid mediated gene transfer: Positively charged liposomes made of cationic lipids appear to be safe, easy to use and efficient for *in vitro* transfer of DNA and antisense oligonucleotides. The highly negatively charged nucleic acids interact spontaneously with cationic liposomes. Already by simple mixing of the polynucleotides with preformed cationic

liposomes a complete formation of DNA-liposome complexes is achieved. However, due to the lack of fusion peptides and cell-specific markers on the liposomal membrane the *in vivo* transfection efficiency is very low and the incubation times are long, wherefore high doses have to be administered in order to achieve a desired effect. Consequently, undesired side-effects may occur since there is evidence that large amounts of cationic lipids can exhibit toxic effects *in vivo*.

Small oligonucleotides are currently being tested as therapeutic agents for the treatment of cancer and as antiviral agents. Only one of the two DNA strands is transcribed to synthesize messenger RNA (mRNA). The DNA strand transcribed into RNA is called the coding strand or sense strand. The complementary, non-coding or antisense strand has the same sequence as the mRNA. When the non-coding strand is transcribed, it produces antisense RNA molecules that are able to bind to target (sense) mRNA. Once the antisense RNA is bound to the sense RNA the resulting RNA duplex molecules cannot be translated and the production of the protein is blocked. Usually, short synthetic antisense oligonucleotides of 18 to 22 bases effectively bind to the mRNA and inhibit mRNA translation. By this mechanism antisense oligonucleotides can stop the proliferation of human cancer cells. Genes that are involved in cancer exert their effect through overexpression of their normal structural proteins. Genes such as c-fos, c-myc, L-myc, N-myc, c-myb, abl, bcr-abl, c-raf, c-erb-2, K-ras may be potential targets for antisense cancer therapy. Antisense oligonucleotides are also an attractive potential alternative to conventional drugs such as, for example, antiviral agents such as, e.g., the antisense oligonucleotides of tat and gag gene of the human immunodeficiency virus (HIV).

Liposomal membranes comprising reconstituted virus envelopes as described in the literature (Stegmann et al.; EMBO J. 6:2651-2659, 1987) may be called virosomes. They usually comprise a phospholipid bilayer containing phosphatidylcholine (PC) and phosphatidylethanolamine (PE) together with viral envelope, e.g., spike proteins embedded in the membrane. The conventional methods to incorporate genetic material into PC,PE-virosomes suffer from the drawback of a rather low efficiency of nucleic acid incorporation.

SUMMARY OF THE INVENTION

The present invention therefore relates to a novel cationic virosome which due to its specific membrane composition may very efficiently be loaded with any desired genetic material comprising long and short chain DNA or RNA, oligodeoxynucleotides, ribonucleotides, peptide nucleic acids (PNA), ribozymes (RNA molecules with enzymatic activities), genes, plasmids and vectors and, thus, convincingly overcomes the drawbacks of the prior art.

The invention further relates to a method for the efficient reconstitution of hemagglutinin of influenza virus A, particularly of strain A/Singapore, into substantially unilamellar cationic lipid vesicles resulting in the formation of cationic virosomes with a mean diameter of approximately 120 - 180 nm and a continuous lipid bilayer, which is substantially free from the disadvantage of leakage seen with many conventional virosome preparations. The structure of the - preferably unilamellar - cationic bilayer membrane is such that the hydrophilic, positively charged heads of the lipids are oriented towards the aqueous phase(s) and the hydrophobic fatty acid tails are oriented towards the center of the bilayer. It could be shown by electron microscopy that the reconstituted viral spike proteins (hemagglutinin) are integrated in the lipid bilayer and extend from the surface of the cationic vesicles (Fig. 1).

The lipid composition of the vesicle membrane comprises cationic and/or polycationic lipids and optionally phospholipids such as phosphatidylethanolamine and phosphatidylcholine. For the most cases it proved advantageous to choose a lipid composition of the membrane comprising - based on total lipids - either

(i) 100 % by weight of cationic and/or polycationic lipids; or

(ii) 90 to 95 % by weight of cationic and/or polycationic lipids and 5 to 10 % by weight of phosphatidylethanolamine; or

(iii) 45 to 90 % by weight of cationic and/or polycationic lipids, 5 to 10 % by weight of phosphatidylethanolamine and 5 to 50 % by weight of phosphatidylcholine.

In a preferred embodiment, the present invention also relates to the irreversible covalent linkage of cell-specific markers to the cationic virosomes including but not being limited to monoclonal antibodies,

antibody fragments such as F(ab')2 and Fab' fragments, cytokines, and/or growth factors, usefule for a selective detection and binding of target cells. They are linked to the vesicle membrane such that they extend to the exterior and exert essentially full functional activity with respect to receptor detection and binding.

Coupling cell-specific markers such as antibody fragments to preformed vesicles as described by, e.g., Martin et al. (J. Biol. Chem. 257: 286-288, 1982) often leads to low and frequently irreproducible coupling yields - a complication that imposes a significant limitation to the targeting strategy. Therefore, according to a preferred embodiment of the present invention the markers are coupled to preformed phosphatidylethanolamine-crosslinker molecules such as, for example, N-[4-(p-maleimido-phenylbutyryl]-phosphatidylethanolamine (MPB.PE) in the presence of a detergent.

In order to achieve the best possible results it proved advantageous to carefully isolate and purify the viral glycoproteins before reconstitution in order to avoid inactivation by either proteolytic digestion or reduction of intramolecular S-S bonds. Accordingly, it is preferred that the conjugated markers, e.g., marker-MPB.PE, be separated from unconjugated phosphatidylethanolamine-crosslinker molecules (e.g., MPB.PE) by affinity chromatography with an activated agarose matrix, preferably with reduced Thiopropyl Sepharose 6B. Aliquots of the purified conjugated markers (phosphatidylethanolamine-crosslinker-marker molecule complexes) are then added to the detergent solution containing the mixture of dissolved membrane lipids, fusion peptides and other desired ingredients, before the cationic virosomes are formed thereof.

It has proven advantageous to carry out the coupling procedure of the bifunctional crosslinker with the phospholipid and the cell-specific marker in a separate process prior to the preparation of the virosomes. This procedure allows to better control and optimize the surface density of the virosome membranes, particularly with respect to the number of cell-specific markers linked thereto. The improved control of the concentration of protein molecules embedded in or linked to the membrane is important in as much as an unbalanced ratio of fusion peptides (e.g., hemagglutinin) and cell-specific markers (e.g., antibody Fab' fragments) on the virosome

membrane may reduce or even destroy their selective properties and - at the extreme - may result in clotting and precipitation of the vesicles.

The use of antibody fragments F(ab')2 and Fab' instead of whole antibody molecules as cell-specific markers is particularly advantageous, because they are far less immunogenic than the whole antibody. Also, the absence of the Fc domain eliminates a range of undesired Fc-mediated biological and immunological activities such as, for example, complement activation via the classical pathway and acute humoral responses eventually resulting - amongst others - in the clearance of attached virosomes from the target cell surface via interaction between the antibody and its Fc-receptor on the target cell.

Unlike known liposomal compositions for delivery of nucleic acids, the present cationic virosomes usually need not fuse with or destabilize the plasma cell membrane to enter the cytoplasm. They are capable of entering the host cells via a two step mechanism: 1. attachment and 2. penetration. In the first step they bind via the fusion peptides (e.g. hemagglutinin) and/or the cell-specific markers to cell receptors, particularly to membrane glycoproteins or glycolipids with a terminal sialic acid, and are then very efficiently incorporated by receptor-mediated endocytosis. In case of virosomes bearing cell-specific markers, e.g., antibody fragments, these markers will additionally recognize antigenic structures on the target cell surface, resulting in an attachment by two different binding mechanisms. Thus, the present cell-specific virosomes exert a selectivity for various cell types owing to their cell-specific markers on the membrane and, simultaneously, a high capability for cell penetration by endocytosis owing to the viral fusion peptide, e.g., hemagglutinin. Virosomes with Fab' fragments that recognize tumor-associated antigens such as TAG72, CEA, 17-1A, CA19-9 or leukemia-associated antigens such as CD10 (CALLA = Common Acute Lymphocytic Leukaemia Antigen) and CD20 will bind selectively to tumor or leukemia cells carrying the mentioned antigens on their cell surface.

In the second step, when entering the host cells via receptor-mediated endocytosis the virosomes get entrapped in endosomes. Subsequently, the pH within the endosomes decreases to about pH 5 - 6, which activates the

hemagglutinin fusion peptide and triggers the fusion of the virosomal membrane with the endosomal membrane. The membrane fusion reaction opens the lipid envelope of the virosomes and liberates the entrapped genetic material into the cytosol. This mechanism considerably improves the chances of the transferred genetic material to reach the nucleus before getting cleared by digestive degradation and/or exocytosis.

DESCRIPTION OF THE INVENTION

The primary objective of the present invention is to provide positively charged lipid vesicles comprising cationic or polycationic lipids and an internal - usually aqueous - space, and further comprising at least one viral fusion peptide embedded or integrated in or covalently linked to the vesicle membrane. The vesicle preferably also comprises at least one cell-specific marker on the membrane. It is a further object of the present invention to provide vesicles having full biological fusion activity, i.e., having essentially the same fusion activity as intact influenza virus. The fusion peptide is a viral glycoprotein such as hemagglutinin or a derivative thereof, or a synthetic fusion peptide being capable of inducing a rapid fusion of said vesicles with the endosomal membranes of the target cells after endocytosis.

The novel vesicles or virosomes are particularly useful to transfer any desired genetic material to target locations, in particular to animal and human cells and tissues *in vitro* and *in vivo*. It is emphasized that the novel virosomes are not only able to penetrate proliferating, i.e., replicating cells but also non-proliferating, i.e., resting cells, which feature makes them widely applicable in the fields of biosciences, pharmacology and medicine, both as a research and/or diagnostic tool and as a medicament. For the use as a medicament, the present virosomes may be part of a pharmaceutical composition which further comprises usual additives and pharmaceutically suitable carriers. It is preferred that the pharmaceutical composition is prepared as an injection solution, but other forms of preparation, e.g., emulsions, cremes, gels, ointments, for topical or systemic administration may be advantageous for some applications.

Therefore, it is also an objective of the present invention to use the present virosomes for the manufacture of a pharmaceutical composition suitable for

the prophylactic and/or therapeutic treament of animal or human individuals who may benefit from such treatment. It is another objective of the present invention to use the present virosomes for the manufacture of a diagnostic kit for *in vitro* and *in vivo* applications.

In one embodiment the present vesicles are obtained by a process comprising an efficient reconstitution of hemagglutinin (HA) of influenza virus A. Accordingly, it is also an object of the present invention to teach a method of preparing cationic virosomes. In a preferred embodiment, the method further comprises the steps of incorporating genetic material into the cationic lipid vesicles. Basically, the method of preparation comprises the following steps:

1) Dissolution of the cationic lipids in a non-ionic detergent, preferably octaethyleneglycol mono-n-dodecylether (OEG, $C_{12}E_8$), together with - preferably purified - viral spike glycoproteins, genetic material desired for delivery and optionally preformed complex molecules made of phosphatidylethanolamine, crosslinker and cell-specific marker; and
2) vesicle formation through - preferably repeated - detergent removal with detergent absorbing micro-carrier beads, preferably polystyrene beads of the SM-2 Biobeads type with a preferred mesh size (wet) of 20-50 (0.84 - 0.30 mm).

In a preferred embodiment of the invention, a suitable bifunctional crosslinker is applied to link the cell-specific marker irreversibly to the vesicle membrane. The cell-specific marker, which is directed to a cell-receptor responsible for the selective binding of the virosome to the cell, is bound to the crosslinker in such a manner that it is still fully biologically active. It is preferred that the crosslinker be employed in the form of a preformed molecule-complex wherein the crosslinker is covalently bound to either phosphatidylethanolamine or to both phosphatidylethanolamine and a cell-specific marker.

Due to the functionally active fusion peptides of the present virosomes the encapsulated material is released to the cytosol of a target cell mainly upon decrease of the endosomal pH (as outlined above). Such controlled release on one hand prolongs the residence time of the delivered material within the

target cell and on the other hand avoids an undesired long stay of the virosomes inside the endosomes and therewith reduces the danger of unspecific degradation of the valuable substances transported by the virosomes.

In still another embodiment, the present invention refers to vesicles where the membrane lipids additionally comprise phosphatidylcholine and phosphatidylethanolamine, which further improves the possibilities of specific virosome design and/or facilitates the anchoring of fusion peptides and/or cell-specific markers to the membrane.

The term "fusion peptide" refers to peptides or proteins capable of inducing and/or promoting a fusion reaction between the virosome membrane and a lipid membrane of the target cell. In most embodiments, it refers to viral spike glycoproteins containing the fusion peptide, particularly to the complete hemagglutinin trimer of viral surface spikes, a monomer thereof, or to one or both cleaved subunits, the glycopeptides HA1 and HA2, containing the functional fusion peptide. In another embodiment of the present invention the term refers to the pure fusion peptide itself, either isolated from natural sources or synthetically produced. In a particularly preferred embodiment of the present invention, these polypeptides containing the fusion peptide refer to influenza hemagglutinins, especially the one of the A-H_1N_1 subtype. The synthetic fusion peptides are preferably selected from the amino acid sequences listed in Table 1 below, wherein the amino acids are identified by their corresponding one letter codes (see also Example 6 and Fig.2 of WO92/13525).

The term "crosslinker" refers to an organic heterobifunctional molecule capable of linking to the surface of vesicles prepared according to this invention and capable of binding polypeptides. In a preferred embodiment of the present invention, this molecule contains a N-hydroxysuccinimide group for coupling to the amino group of phosphatidylethanolamine and a maleimide group for conjugation of monoclonal antibody fragments, such as succinimidyl 4-(N-maleimidomethyl)-cyclohexane-1-carboxylate, m-maleimidobenzoyl-N-hydroxysuccinimide ester, m-maleimidobenzoyl-N-hydroxysulfosuccinimide ester, succinimidyl 4(p-maleimidophenyl)-butyrate, sulfosuccinimidyl 4(p-maleimidophenyl)butyrate; or

it contains a N-hydroxysuccinimide group and a photoreactive azido group for coupling to cytokines, such as N-hydroxysuccinimidylsuberate (NHS-SA), N-hydroxysuccinimidyl-4-azidobenzoate (HASAB), N-succinimidyl-6-(4'-azido-2'-nitrophenylamino)hexanoate (SANPAH), N-sulfosuccinimidyl-6-(4'-azido-2'-nitrophenylamino)hexanoate.

Table 1:

C C C G L F G	A I A G F I E N G W E G M I D G	W Y G
G L F G	A I A G F I E N G W E G M I D G	W Y G C C C
C C C G L F G	A I A G F I E N G W E G M I D G	
G L F G	A I A G F I E N G W E G M I D G	C C C
C C C G L F E	A I A G F I E N G W E G M I D G	
G L F E	A I A G F I E N G W E G M I D G	C C C
C C C E L F G	A I A G F I E N G W E G M I D G	
E L F G	A I A G F I E N G W E G M I D G	C C C
C C C L F G	A I A G F I E N G W E G M I D G	
L F G	A I A G F I E N G W E G M I D G	C C C
C C C	P P G A V I G T I A L G V A T A A G I T	
P P G A V I G T I A L G V A T A A G I T		C C C
C C C	P A G V V I G L A A L G V A T A A G V T	
P A G V V I G L A A L G V A T A A G V T		C C C
C C C	P I G A I I G G V A L G V A T A A G I T	
P I G A I I G G V A L G V A T A A G I T		C C C

It is preferred that the crosslinker be used in the form of a preformed molecule complex of crosslinker and lipid, notably of crosslinker and

phosphatidylethanolamine, or of lipid plus crosslinker plus cell-specific marker.

The term "cell-specific" protein or marker refers to a protein capable of linking to the crosslinker or crosslinker-lipid complex, respectively, and further being capable of binding to the receptor of target cells. In a preferred embodiment of the present invention, this molecule refers to a cell receptor-specific compound such as a monoclonal antibody, an antibody fragment, a cytokine or a growth factor. The cell-specific marker provides for selective detection and binding of target cells and thus improves the action of the fusion peptide concomitantly present in the virosomal membrane. The preferred antibody fragments comprise the $F(ab')_2$ and Fab' fragments, while the cell-specific markers further comprise interleukins and other cytokines, particularly the ones listed in Table 2 below.

Table 2

Cytokines (international abbreviations)			
BDNF	IFNα	MIP-1α	PDGF
CNTF	IFNβ	MIP-1β	PF-4
EGF	IFNγ	MIP-2	RANTES
Epo	IL-1 to IL-15	NGF	SCF
FGF	LIF	NT-3	TGFα
G-CSF	LT (TNFβ)	NT-4	TGFβ
GM-CSF	MCP-1 to MCP-3	OSM	TNFα
I-309/TCA-3	M-CSF	PBP	Tpo
γIP-10	MIF	PBSF	jVEGF

The term "cationic lipid" as used herein refers to an organic molecule that contains a cationic component and a nonpolar tail, a so-called head-to-tail amphiphile, such as N-[(1,2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA) (Felgner et al.; Proc Natl Acad USA 84:7413-7417, 1987), N-[1,2,3-dioleoyloxy)-propyl]-N,N,N-trimethylammoniummethyl-sulfate (DOTAP); or N-t-butyl-N´-tetradecyl-3-tetradecylaminopropion-amidine (Ruysschaert et al.; Biochem. Biophys. Res. Commun. 203:1622-1628, 1994). Unless explicitly mentioned otherwise, the term also includes the below defined polycationic lipids.

The term "polycationic lipid" refers to an organic molecule that contains a polycationic component and a nonpolar tail such as the lipospermine: 1,3-dipalmitoyl-2-phosphatidylethanolamido-spermine (DPPES) and dioctadecyl-amidoglycyl spermine (DOGS) (Behr et al. ; Proc. Natl. Acad. USA 86:6982-6986, 1989); 2,3-dioleoyloxy-N-[2(sperminecarboxamido) ethyl]-N,N-dimethyl-1-propaneaminium trifluoroacetate (DOSPA); 1,3-dioleoyloxy-2-(6-carboxy-spermyl)-propylamide (DOSPER); N,N,N´,N´-tetramethyl-N,N´-bis(2-hydroxyethyl)-2,3-dioleoyloxy-1,4-butanediammonium iodide (THDOB).

The terms "nucleic acid" or "genetic material" as used herein comprise short chain DNA or RNA, deoxyribonucleotides, oligodeoxyribonucleotides, oligodeoxyribonucleotide selenoates, oligodeoxyribonucleotide phosphorothioates (OPTs), oligodeoxyribonucleotide phosphoramidates, oligodeoxyribonucleotide methylphosphonates, peptide nucleic acids (PNAs), ribonucleotides, oligoribonucleotides, oligoribonucleotide phosphorothioates, 2'-OMe-oligoribonucleotide phosphates, 2'-OMe-oligoribonucleotide phosphorothioates, ribozymes (RNA molecules with enzymatic activities), genes, plasmids and vectors (cloning vehicles).

The term "virosomes" as used herein refers - in its simplest form - to liposomal vesicles with a bilayer membrane comprising cationic lipids and an internal - preferably aqueous - space, wherein the membrane further contains viral proteins, in particular viral glycoproteins. In the preferred embodiment, the viral proteins comprise at least one fusogenic peptide or protein having full biological fusion activity, particularly the spike glycoprotein hemagglutinin and/or neuraminidase of influenza A (e.g., A/Singapore) virus. It shall be understood that the viral proteins also encompass synthetically produced amino acid sequences corresponding to or equal to the fusion peptide of influenza virus as herein described. The membrane lipids comprise the cationic lipids defined above but may optionally further comprise other natural and/or synthetic lipids, preferably phospholipids such as phosphatidylcholine (PC) and phosphatidylethanol-amine (PE).

Although the cationic virosomes of the present invention may in many cases - notably for *in vitro* cell culture experiments - successfully be applied without cell-specific markers on the membrane, it is particularly preferred

for *in vivo* applications that they further comprise at least one cell-specific marker on the membrane as hereinbefore defined. The mean diameter of the vesicles is in the range of 120 - 180 nm, as determined by electron microscopy and dynamic light scattering.

The term "full (biological) fusion activity" as used herein shall express that the virosomes of the present invention comprising reconstituted viral proteins in the vesicle membrane have essentially the same fusion activity towards target cells as the intact virus from which they are usually reconstituted. Preferably, the comparison of the cationic virosomes' fusogenicity is drawn to intact influenza A virus. The fusion activity is measured according to known procedures, particularly as reported by Hoekstra et al. (Biochemistry 23:5675-5681,1984), or Lüscher et al. (Arch. Virol. 130:317-326, 1993).

Before antisense technology can therapeutically or prophylactically be applied to a patient in need thereof a number of technical problems, particularly relating to the development of a suitable carrier system, need to be resolved beforehand. For instance, genetic material such as, e.g., antisense oligonucleotides, can be unstable and break down or be otherwise more or less inactivated before it reaches the target cells and it may thus be necessary to use large quantities of such material entrapped in conventional cationic liposomes. Due to these large amounts a question arises about the potential toxicity in the human or animal body.

By using the cationic virosomes of the present invention as carriers for genetic material these problems can be successfully overcome and undesired side effects due to toxicity can be prevented or at least considerably decreased. This beneficial effect is achieved because the present cationic virosomes have - compared to liposomes or virosomes known hitherto - a far higher activity and efficiency of up to a factor of 1'000-20'000 for the transfer of entrapped genetic material such as antisense oligonucleotides into target cells. As a consequence, it is practically impossible to compare the performance of conventional virosomes or cationic liposomes with the performance of the present cationic virosomes.

BRIEF DESCRIPTION OF THE FIGURES

Fig.1 shows a micrograph of DOTAP virosomes with viral spike proteins.

Fig.2 shows the pH-induced fusion activity of octadecyl rhodamine B labeled DOTAP-virosomes with model liposomes.

Fig.3 shows DOTAP-virosomes with encapsulated antisense FITC-OPT incorporated into human small cell lung cancer cells.

Fig.4 and Fig.5 show the extraordinary uptake and transfection efficiency of antisense-L-myc-DOTAP-virosomes into human small cell lung cancer cells.

Fig.6 shows the incubation of different human small cell lung cancer cells with Antisense-L-myc virosomes.

Fig.7a, 7b show the transfection efficiency of pRSVcat-DOTAP virosomes for Jurkat cells.

Fig. 8: Fusion of DOTAP-virosomes with phospholipid-liposomes. Fusion was measured with the R18 assay at 37°C.

Fig. 9: Thymidine incorporation into virosome-treated NCI-H209 cells. 75 µl of virosomes containing 200 picomol of either antisense, sense, or msc FITC-OPT and 625 µl of fresh medium containing 0.5 µCi ^{14}C-thymidine were added to $5x10^4$ cells/ml per well.

Fig. 10: Dose-dependent inhibition of thymidine incorporation into NCI-H209 cells upon addition of virosomes containing antisense-L-myc-OPT. NCI-H209 cells were incubated at an initial cell concentration of 1×10^5 per well and per ml. Values are the means ± standard deviations of three experiments.

Fig. 11: Incubation of different human small cell lung cancer cell lines with 75 µl of antisense-L-myc virosomes. L-myc oncogen expression decreases in the cell lines as indicated: H82 < H510A < H209. The virosomes of lot 1 contained a smaller amount of antisense-L-myc than the ones of lot 2.

Fig. 12: Transfection of Sp2 cells by two differently produced virosome preparations.

Fig. 13: Transfection of P3/NS1 cells by two differently produced virosome preparations.

Fig. 14: Transfection of NIH/3T3 cells by two differently produced virosome preparations.

Figs. 15, 16, 17: Cell growth of KG1 cells upon treatment with sense and antisense c-myb-DOTAP virosomes. Addition of 25, 50 or 100 µl of virosome solution containing 18, 36, or 72 pmol OPT, respectively. Values are the means ± standard deviations of three experiments.

Fig. 18: Cell growth of CEM-C3 cells upon treatment with DOTAP-virosomes at the addition of 50 µl of sense and antisense c-myb virosome solution. Values are the means ± standard deviations of three experiments.

In order that the invention described herein may be more fully understood, the following examples are set forth. The examples are for illustrative purposes only and are not to be construed as limiting this invention in any respect.

Example 1

Preparation of a cationic lipid vesicle with fully fusion active viral hemagglutinin trimers from influenza virus containing encapsulated antisense L-myc-FITC(= fluorescein labeled)-oligodeoxynucleotides.

Preparation of DOTAP virosomes and DOTAP-Phosphatidylcholine (PC)-virosomes

Hemagglutinin (HA) from the A/Singapore/6/86 strain of influenza virus was isolated as described by Skehel and Schild (1971), Proc.Natl.Acad.Sci.USA 79:968-972. In short, virus was grown in the allantoic cavity of hen eggs, and was purified twice by ultracentrifugation in a sucrose gradient. Purified virus was stabilized in a buffer containing 7.9 mg/ml NaCl, 4.4 mg/ml trisodiumcitrate·2H_2O, 2.1 mg/ml 2-morpholinoethane sulfonic acid, and 1.2 mg/ml N-hydroxyethyl-piperazine-N'-2-ethane sulfonic acid pH 7.3. 53 ml of the virus suspension containing 345 µg HA per ml were pelletted by ultracentrifugation at 100'000 x g for 10 minutes. 7.7 ml of a buffered

detergent solution containing 145 mM NaCl, 2.5 mM HEPES and 54 mg/ml of the non-ionic detergent octaethyleneglycol monododecylether (OEG = $C_{12}E_8$), pH 7.4, were added to the influenza virus pellet. The pellet was completely dissolved by using ultrasonication for 2 minutes at room temperature. The solution was subjected to ultracentrifugation at 100'000 x g for 1 hour. The obtained supernatant contained the solubilized HA trimer (1.635 mg HA/ml) and trace amounts of neuraminidase. 6 mg of DOTAP were added to 3.7 ml of supernatant (6 mg HA) and dissolved. The solution was sterilized by passage through a 0.2 µm filter and then transferred to a glass container containing 1.15 g of sterile microcarrier beads, preferably Biobeads SM-2. The container was shaken for 1 hour by using a shaker REAX2 from Heidolph (Kelheim, Germany). When necessary, this procedure was repeated up to three times with 0.58 mg of Biobeads. After these procedures a slightly transparent solution of DOTAP virosomes was obtained.

For the production of DOTAP-PC virosomes 3 mg of DOTAP and 3 mg of PC were added to the supernatant containing 6 mg HA, and dissolved. The subsequent steps were the same as described for the DOTAP virosomes.

Preparation of virosomes with synthetic fusion peptide

One possibility of preparing cationic virosomes carrying synthetic fusion peptides in the membrane comprises the following steps:

1. Activation of phosphatidylethanolamine (PE) by the crosslinker N-[γ-maleimidobutyryloxy]succcinimide ester (GMBS) in a reaction

PE + GMBS → MBS-PE + N-Hydroxysuccinimid,

as described by Martin et al., Irreversible coupling of immunoglobulin fragments to preformed vesicles; J. Biol. Chem. 257:286-288 (1982).

2. Preparation of lipid vesicles with activated PE (GMB-PE), wherein 20% Phosphatidylcholine, 70 % DOTAP and 10% GMB-PE are dissolved in a buffered detergent solution as described above for the HA containing DOTAP virosomes. The subsequent steps of preparing the lipid vesicles are the same as described above for the DOTAP virosomes.

3. Coupling of the synthetic fusion peptide to the lipid vesicles, wherein

a peptide comprising 20 amino acids is used having the amino acid sequence G-L-F-E-A-I-A-G-F-I-E-N-G-W-E-G-M-I-D-C, and which contains a free amino group at the N-terminus and an amide group at the C-terminus. Since the amino acid at the C-terminus is cysteine, there is a free thiol group available for the coupling of the peptide to the GMP-PE in the membrane of the lipid vesicles.

For carrying out the coupling reaction:

$PE\text{-}GMB_{membrane}$ + HS-Cys-Peptide → $PE\text{-}GMB_{membrane}$-S-Cys-Peptide

a solution of freshly prepared lipid vesicles in a buffer (40 mM citric acid, 35 mM disodium phosphate, 100 mM NaCl, and 2 mM EDTA, pH 5.5) is mixed with the peptide solution in the same buffer. The mixture is gently stirred under nitrogen atmosphere overnight at 4°C. Lipid vesicles are separated from unconjugated peptides by gel filtration on a High Load Superdex 200 column.

As an alternative, the fusion peptides may also be coupled to the lipid vesicles my means of preformed PE-crosslinker-peptide complexes as described below in this example for the Fab' fragments.

Incorporation of phosphorothioate oligodeoxyribonucleotides into DOTAP virosomes

The antisense and sense oligodeoxyribonucleotide phosphorothioates (OPTs) of the L-*myc* gene were used as an example for the demonstration of the high efficiency of cationic virosomes in transfection. 5'-FITC-OPTs were synthesized via phosphoramidite chemistry (Microsynth GmbH, Balgach, Switzerland). The pentadecamer (5'-FITC-GTAGTCCATGTCCGC-3') and the pentadecamer (5'-FITC-GCGGACATGGACTAC-3') were used as the antisense OPT and sense OPT, respectively. A mixed sequence control (msc) OPT consisting of the same length of nucleotides as antisense and sense OPTs was synthesized.

1 ml of DOTAP virosomes or DOTAP-PC virosomes was added to each of

a) 2 mg of antisense FITC-OPT (1.3 µmol),

b) 3.4 mg sense FITC-OPT (1.3 µmol) and

c) 3.1 mg msc FITC-OPT (1.3 µmol).

The FITC-OPTs were dissolved and the solutions were then treated by sonication for 2 minutes at 26°C. Non-encapsulated FITC-OPTs were separated from the virosomes by gel filtration on a High Load Superdex 200 column (Pharmacia, Sweden). The column was equilibrated with sterile PBS. The void volume fractions containing the DOTAP virosomes with encapsulated FITC-OPT were eluted with PBS and collected. Virosome-entrapped FITC-OPT concentrations were determined fluorometrically after the virosomes were fully dissolved in 0.1 M NaOH containing 0.1% (v/v) Triton X-100. For calibration of the fluorescence scale the fluorescence of empty DOTAP-virosomes that were dissolved in the above detergent solution was set to zero.

Coupling of Fab'-fragments to virosomes by means of preformed phosphatidylethanolamine-bifunctional crosslinker molecule complexes

3 mg of freshly reduced Fab' from murine monoclonal anti-CD10- (Anti-CALLA) antibody, dissolved in 2.8 ml of a citric acid buffer solution (100 mM NaCl, 40 mM citric acid, 35 mM $Na_2HPO_4.2H_2O$, 2 mM EDTA, pH 5.5) were added to a solution of 0.524 mg of N-[4-(p-maleimido-phenyl)-butyryl] phosphatidylethanolamine (MPB.PE) in 215 µl of citric acid buffer containing 0.5% of n-octyl-oligo-oxyethylene. The mixture was then incubated under nitrogen for 16 h at 4°C with gentle stirring. After incubation the non-coupled MPB.PE was removed by a batch of 400 µl of freshly reduced wet Thiopropyl Sepharose 6B (Pharmacia, Sweden). The mixture was incubated for 4 h at room temperature. The Thiopropyl Sepharose 6B was removed by centrifugation and the resulting solution neutralized to pH 7.0. The neutralized solution was supplemented with OEG (54 mg/ml).

The solutions prepared as described above were added to the solutions for the preparation of DOTAP virosomes. The Fab'-MPB.PE molecules are inserted into the lipid bilayer during the formation of virosomes.

Electron Microscopy Observations

Micrographs of DOTAP virosomes confirm the preferred unilamellar structure of the vesicles with an average diameter of approximately 120 to 180

nm as determined by laser light scattering. The HA protein spikes of the influenza virus are clearly visible (Fig.1).

Determination of the Fusion Activity of DOTAP Virosomes

The fusion activity of the present DOTAP virosomes was measured by the quantitative assay based on fluorescence dequenching described by Hoekstra et al. (1984), Biochemistry 23:5675-5681 and Lüscher et al. (1993), Arch. Virol. 130:317-326. The fluorescent probe octadecyl rhodamine B chloride (R18) (obtained from Molecular Probes Inc., Eugene, USA) was inserted at high densities into the membrane of DOTAP virosomes by adding the buffered OEG ($C_{12}E_8$) solution containing DOTAP and HA to a thin dry film of the fluorescent probe, followed by shaking for 5 to 10 minutes for dissolving the probe, then continuing as described above under "Preparation of a cationic lipid vesicle...". Dilution of the quenching rhodamine was observed by incubation of the rhodamine-labeled DOTAP virosomes with model liposomes (ratio of DOTAP: liposomal phospholipid = 1:20). The fluorescence was measured by a Perkin-Elmer 1000 spectrofluorimeter at 560 and 590 nm excitation and emission wavelengths, respectively. Fig.2 shows the pH-induced fusion reaction of DOTAP virosomes expressed as percent of fluorescence dequenching (% FDQ).

Cellular uptake of encapsulated Antisense-L-myc-FITC-OPT

It proved very useful to label the OPT with fluorescein to study the mechanism of cellular uptake of DOTAP virosomes.
Human small cell lung cancer cells (ATCC-NCI-H209) which express high levels of L-myc gene (Nau et al. 1985; Nature 318, 69-73) were grown in 2-well tissue culture chamber slides (Nunc, Naperville, IL 60566, USA). 50 µl of FITC-OPT-virosomes were added to the cells. They were incubated for 5, 15, and 30 min at 37°C, washed twice with PBS and then examined by fluorescence microscopy. DOTAP virosomes with encapsulated antisense FITC-OPT were rapidly incorporated into the cells as can be seen in Fig.3.

Examination of the biological effect of Antisense-L-myc-FITC-OPT-DOTAP virosomes measured by the thymidine incorporation method

Human small cell lung cancer cells (ATCC-NCI-H209 American Type Culture Collection, Rockville, USA) were cultured in 24-well Costar plates at an initial concentration of 1 x 10^5 per well and per ml. After an incubation of 24 hours, medium was removed and 625 µl of fresh medium containing 0.5 µCi ^{14}C-thymidine (prepared from [2-^{14}C] thymidine, 52.0 mCi/mmol; Amersham, England) and 75 µl of DOTAP virosomes containing 0.2 nmol of either antisense, sense, or msc FITC-OPTs were added. The cultures were gently shaken at very slow agitation for 1 hr at 37°C and then transferred to the incubator. After 48 hours the cell suspensions were removed, transferred to centrifuge vials, and centrifuged. Obtained cell pellets were washed twice. When the cells could not sufficiently be dispersed into a single cell suspension, they were exposed briefly to a trypsin/EDTA solution. Cell pellets were dissolved in 1.5 ml of 0.1 M NaOH/Triton-X-100 (0.1%) solution. 3 ml of liquid scintillation cocktail (Ready Protein +, Beckman, Fullerton, CA, USA) were added to 1 ml of solution. ^{14}C-radio-activity was counted in a liquid scintillation counter (Beckman, Fullerton, CA, USA).

Figures 4 and 5 clearly demonstrate the extraordinary uptake and transfection efficiency of antisense-L-myc-DOTAP virosomes.

Fig. 6 demonstrates that cells that do not express L-myc are not influenced or inhibited by the antisense-L-myc virosomes. Also, empty virosomes did not show any effects on cancer cells and normal cells. It appears therefore that an anti-cancer therapy with antisense OPT encapsulated in the present virosomes may have a great potential, particularly because of their lack of the hereinbefore mentioned disadvantages of conventional cationic liposomes.

Example 2

Preparation of a cationic lipid vesicle with fully fusion active viral hemagglutinin trimers from influenza virus containing the encapsulated vector pcDNA3 with human IL-6 gene cloned into the polylinker site (= pcDNA3-IL-6)

Preparation of DOTAP virosomes and incorporation of pcDNA3-IL-6

pcDNA3 (Invitrogen Corporation, San Diego, USA) is a 5.4 kb vector designed for high-level stable and transient expression in eukaryotic hosts. HA was isolated and purified as described in Example 1.

4 mg of DOTAP were dissolved in 0.5 ml of the buffered detergent solution containing 145 mM NaCl, 2.5 mM HEPES and 54 mg/ml of OEG (= $C_{12}E_8$), pH 7.4, and added to 2 ml of supernatant containing 4 mg HA. To the resulting mixture 100 µg of pcDNA3-IL-6 were added and dissolved. The solution was subjected to ultrasonication for 30 seconds. OEG was removed by Biobeads as described in Example 1.

Transfection of DOTAP virosomes loaded with pcDNA-IL-6 into murine myeloma cells

The obtained solution comprising the pcDNA-IL-6 loaded DOTAP virosomes was diluted 1:1000 with PBS. 20 µl and 50 µl of this solution containing 1 ng and 2.5 ng pcDNA-IL-6, respectively, were added to 2 x 10^6 myeloma cells (P3/NSI/1-Ag4-1; American Type Culture Collection, Rockville, USA). After 48 h incubation the supernatants of the cell cultures were tested for human IL-6 by an ELISA assay. A content of 20 to 45 pg IL-6 per ml was measured.

Comparison of transfection efficiency of pcDNA-IL-6 loaded DOTAP virosomes with pcDNA-IL-6 loaded DOTAP liposomes

No IL-6 was found in myeloma cell cultures transfected with conventional DOTAP liposomes (which are devoid of viral fusion peptides) containing the same amount of pcDNA-IL-6 as the DOTAP virosomes. In order to obtain the same transfection results as with the pcDNA-IL-6 loaded DOTAP virosomes it was necessary to increase the amount of the pcDNA-IL-6 loaded DOTAP liposomes by a factor of one thousand (1000) !!

Example 3: Preparation of a cationic lipid vesicle with fully fusion active viral hemagglutinin trimers from influenza virus containing the encapsulated vector pRSVcat

Preparation of DOTAP virosomes and incorporation of pRSVcat

The expression vector pRSVcat (from ATCC, Rockville, USA) contains the CAT gene which codes for the chloramphenicol acetyltransferase (CAT).

The enzyme catalyzes the transfer of an acetyl group from acetyl-CoA to the 3´-hydroxy position of chloramphenicol. CAT vectors are useful for monitoring transfection efficiency in general.
pRSVcat was encapsulated into DOTAP virosomes under the conditions described in Example 2.

Transfection of DOTAP virosomes loaded with pRSVcat into Jurkat cells
Jurkat cells (10^6 cells/ml) were incubated with different amounts of pRSVcat-loaded DOTAP virosomes (0.0001 µl - 25 µl). After 48 hours incubation at 37 °C the CAT activity in the Jurkat cells was measured by the CAT-ELISA assay (Boehringer Mannheim, Germany).
Figures 7a and 7b demonstrate that a maximum transfection is achieved by addition of 0.01 µl of DOTAP virosomes. Contrary to the aforementioned, the addition of 0.01 µl of pRSVcat-loaded DOTAP liposomes to Jurkat cells under the same incubation conditions did not result in any detectable CAT activity.

Example 4: Uptake of virosomes by cells

Entry of virosomes into target cells can be divided into two distinct steps:

1. Attachment
2. Penetration.

Attachment involves binding of the virosomes via HA to the cell receptors which are membrane glycoproteins or glycolipids with a terminal sialic acid. In case of specific virosomes Fab' fragments will additionally recognise antigenic structures on the target cell surface, resulting in an attachment to target cells by two different binding mechanisms. Thus, specific virosomes exert a selectivity for special cell types. Virosomes with Fab' fragments that recognise tumor associated antigens such as TAG72, CEA, 17- 1A, CA19-9 or leukemia associated antigens such as CD10 (CALLA) and CD20 will bind selectively to tumor or leukemia cells carrying the mentioned antigens on their cell surface. The hemagglutinin glycoproteins are carefully isolated and purified. There is no inactivation either by proteolytic digestion or by reduction of its intramolecular disulfide (-S-S-) bonds.

Penetration involves entry of virosomes into the cells by receptor-mediated endocytosis. The virosomes get trapped in endosomes. The acidic pH (5-6)

within the endosomes triggers fusion of the virosomal membrane with the endosomal membrane. The fusion is mediated by the viral spike glycoprotein hemagglutinin (HA). The membrane fusion reaction in the endosome liberates the virosome from its lipid envelope and provides access for the encapsulated drugs to the cytosol. Fusion activity of these virosome-preparations were tested by fluorescence dequenching. Virosomes were labelled with the fluorescent probe octadecyl rhodamine B (R18) and the fusion activity of HA was monitored as fluorescence dequenching due to the dilution of the probe from the virosomal into a liposomal target membrane. Fig. 8 shows the fluorescence observed upon addition of DOTAP-virosomes, labeled with R18, to phospholipid-liposomes. The fluorescence started to increase rapidly indicating an intact HA mediated fusion.

Time dependent uptake by cells

The uptake of virosomes was measured by incubation of cells with ^{14}C-labeled virosomes. P3/NS1 cells at a concentration of $1x10^5$ /ml were incubated with 40 µl of virosomes at 37°C for 5, 10, 15, 20 and 30 minutes. After washing, the cells were lysed and the amount of ^{14}C-labeled virosomes was measured. As can be seen in Table 3, the cellular uptake is very fast: During the first five minutes 10% of the virosomes were incorporated. Longer incubation times did not enhance the uptake any further. 1 ml of virosome solution contained approximately 10^{11} - 10^{12} virosomes, hence 4'000 - 40'000 virosomes per cell were incorporated within 5 minutes.

Table 3

Incubation Time [min]	dpm
5	6414
10	6832
15	6096
20	6610
30	6626

Example 5: Antisense strategies in the treatment of cancers

So-called "antisense" oligodeoxynucleotides (ODN) are short nucleotide sequences of DNA synthesized as reverse complements of the desired

mRNA target's nucleotide sequence. By formation of the RNA-DNA duplex translation of the message is prevented and the destruction of the molecule by RNase H is promoted. Delivery of ODN targeting oncogene-encoded mRNA to cancer cells may be associated with inhibition of cell proliferation and, in some circumstances, cell death.

Antisense ODN have a great potential as therapeutic agents. Many preclinical animal studies as well as clinical trials of Phases I-III have shown that antisense ODN directed against oncogenes and viral genes are therapeutically active.

Transfer of functional DNA molecules into cells by DNA-loaded liposomes or conventional virosomes is not very efficient. Therefore virosomes with a positively charged lipid bilayer (cationic) were developed for transfer of genetic material. The positively charged lipid bilayer interacts with nucleic acids and causes them to concentrate within the vesicles formed.

Antisense-L-myc-virosomes

The L-myc gene, first discovered in a small cell lung cancer (SCLC) cell line, is frequently amplified and overexpressed in SCLC. 5'-FITC-phosphorothioate oligodeoxyribonucleotides (OPT) were synthesized via phosphoramidite chemistry (Microsynth GmbH, Balgach, Switzerland). The pentadecamer (5'-FITC-GTAGTCCATGTCCGC-3') and the pentadecamer (5'-FITC-GCGGACATGGACTAC -3') were used as the antisense OPT and sense OPT, respectively. A mixed sequence control (msc) OPT consisting of the same length of nucleotides as antisense and sense OPT was synthesized. The antisense OPT covering the translational initiation site acts by inhibiting ribosomal translation of the target mRNA. Antisense-L-myc-phosphorothioate oligodeoxyribonucleotides were encapsulated into the virosomes. The antiproliferative effect of virosome-encapsulated L-myc antisense DNA in the SCLC cell lines H209, H510, and H82 was evaluated. Antisense-L-myc virosomes were added to the cells of human small cell lung cancer cell lines. Sense-L-myc virosomes and msc (mixed sequence control)-virosomes were used as controls (Fig. 9.

Antisense-L-myc virosomes were 20'000-fold more active than non-encapsulated antisense OPT. To induce the same effects as seen in Fig. 10

concentrations of non-encapsulated L-myc-antisense OPT in the range of micromoles had to be added to the cell cultures. Hence, cationic virosomes are far more efficient in the delivery of ODN than the cellular uptake of non-encapsulated ODN and also more efficient than cationic liposomes.

The growth-inhibitory effect of antisense-L-myc virosomes correlated with levels of L-myc expression in the three SCLC cell lines, H209, H510, and H82. From Fig. 11 it was concluded that those cells that do not express the L-myc gene are not influenced by antisense-L-myc virosomes. Empty cationic virosomes did not show any or only minor effects on normal cells and cancer cells. Since the L-myc gene is frequently amplified and overexpressed in SCLC and very restricted and low-level expressed in human adult tissues, L-myc might be a good target for an antisense virosome therapy.

Example 6: Non-infectious transfer of plasmid-based vectors for gene therapy: transfection of vectors for mammalian expression by cationic virosomes

Current approaches to cancer gene therapy use plasmid-based vectors to express suitable target genes in human cancer cells either *ex vivo* or *in vivo*. The following therapeutic gene targets are evaluated: Susceptibility genes such as herpes simplex virus thymidine kinase (HSV-TK) genes (Moolten FL; Cancer Res. 46:5276-5281,1986); genes which target the immune system to eliminate cancer cells such as cytokine genes (Tepper RI et al.; Cell 57:503-512, 1989), genes coding for costimulatory molecules (Townsend SE et al.; Science 259:368-370, 1993), foreign histocompatibility genes (Plautz GE et al.; Proc Natl Acad Sci USA 90: 4645-4649, 1993); and replacement of wild-type tumor suppressor genes such as p53 (Chen PL et al.; Science 250:1576-1580, 1990).

Because of certain limitations of currently used viral-based vectors for gene therapy such as, for instance, lack of specificity in targeting tumor cells for gene transfer, and because of safety concerns regarding the possible induction of secondary malignancies and the possibility of recombination to form replication competent virus, a non-infectious gene transfer technology for in **vivo** gene delivery of plasmid-based expresssion vectors needed to be

developed. The use of the herein disclosed cationic virosomes is a promising alternative of a non-infectious, receptor-mediated gene transfer technology.

The typical transfection efficiencies by using commercially available lipids are between 5-50%. Not only provide virosomes higher transfection efficiency than commercially available liposomes but the entrapment of DNA into virosomes results also in stable transformation of cells.

Human interleukin 6 (IL-6) gene was cloned into the polylinker site of pcDNA3, a 5.4 kb vector designed for high-level stable and transient expression in eukaryotic hosts. The vector contains the neomycin resistance marker, expressed from the SV40 early promoter for the selection of stable transformants in the presence of G418.

Encapsulation of the vector was performed by 3 different methods:

1. Dialysis: Plasmids were encapsulated during formation of virosomes. Detergent Octyl-POE (from Alexis Corp., Laeufelfingen, Switzerland) was removed by dialysis.
2. Biobeads: Plasmids were encapsulated during formation of virosomes. Detergent OEG was removed by Biobeads.
3. Ultrasonication: Plasmids were encapsulated by DOTAP and the obtained DOTAP-liposomes were fused with DOTAP-virosomes by ultrasonication.

^{14}C-thymidine-labeled pcDNA3-cIL-6-DNA was produced for measuring the amount of encapsulated plasmid.

Method of encapsulation	Amount of encapsulated plasmid
Dialysis (1)	0.02 µg DNA per µl of virosomes
Biobeads (2)	0.009 µg DNA per µl of virosomes
Ultrasonication (3)	0.04 µg DNA per µl of virosomes

Sp2/0-Ag14 cells (Hybrid, non-secreting, mouse; ID-No: ATCC CRL-1581; herein termed Sp2), P3/NSI/1-Ag4-1 cells (Non-secreting myeloma, mouse; ID-No: ATCC TIB-18; herein termed P3/NS1) and NIH/3T3 cells (Embryo, contact-inhibited, NIH Swiss mouse; ID-No: ATCC CRL-1658) at a cell concentration of 1 x 10^5 in 1 ml medium were transfected by the virosome

preparations (1) - (3). Ten days after transfection the amounts of expressed IL-6 were measured by ELISA (Fig. 12, Fig. 13, Fig. 14). All cell lines are available from ATCC, 12301 Parklawn Drive, Rockville, Maryland, USA.

Transfected Sp2 and P3/NS1 cells were selected twice by G418. After 2 months of culturing the production of IL-6 was measured again. The values are listed in Table 4.

Table 4: Production of IL-6 by transfected cells after re-selection with G418

Cell line		Method of prepa-ration	Number of cells per ml	Total number of cells	IL-6 [pg/ml]	Total amount of IL-6 [pg]	IL-6 [pg/10^6 cells]
P3/ NS1	Pellets of cells	Dialysis	1.2 x 10^6	6.0 x 10^6 3 ml buffer	277	831	138
		Biobeads	1.6 x 10^6	7.8 x 10^6 4 ml buffer	32	128	16
		Ultrasoni cation	1.54 x 10^6	8.0 x 10^6 4 ml buffer	289	1156	144
P3/ NS1	Super-natant	Dialysis		5 ml supern.	8	40	6.7
		Biobeads		4.9 ml supern	4	20	2.5
		Ultrasoni cation		5.2 ml supern.	25	130	16.2
Sp2	Pellets of cells	Dialysis	2.25 x 10^6	1.13 x 10^7 5.5 ml buffer	> 1200	> 6600	> 584
		Biobeads	1.8 x 10^6	9.5 x 10^6 in 4.5 ml buffer	232	1044	110
		Ultrasoni cation	2.8 x 10^6	1.37 x 10^7 7 ml buffer	680	4760	347

Table 4 continued

Sp2	Super-natant	Dialysis		5 ml supern.	268	1340	119
		Biobeads		5.2 ml supern.	8	42	4.4
		Ultrasoni cation		4.9 ml supern.	651	3190	233

The volume of lysis buffer added to the cell pellets was adjusted so that a cell number of ca. 2 x 10^6 per ml was obtained.

Example 7: Antisense strategies in the treatment of leukemias

The most common genetic abnormality in human leukemias is the Philadelphia Chromosome (Ph^1) translocation. The translocation of the protooncogene abl from chromosome 9 to the breakpoint cluster region (bcr) on chromosome 22 results in the formation of bcr-abl hybrid genes. The abl protooncogene normally encodes a protein with tyrosine kinase activity which is augmented in cells carrying bcr-abl hybrid genes. The bcr-abl transcripts are found in the vast majority of chronic myelogenous leukemia (CML) patients and in Ph^1 acute lymphocytic leukemia patients. The targeting of bcr-abl genes in CML is clearly the most rational therapeutic procedure. Synthetic ODN complementary to the junction of bcr-abl transcripts produced from the splicing of either the second or third exon of the bcr gene to the second exon of c-abl were shown to suppress Philadelphia 1 leukemic cell proliferation in vitro and to spare the growth of normal marrow progenitors (Szczylik C et al.; Science 253:562-565, 1991). However, the bcr-abl antisense therapy is restricted to CML patients.

Another molecular target for antisense therapy is the myb gene. Myb, the encoded product of the protooncogene c-myb, functions as a DNA binding specific transcription factor. It is preferentially expressed in hematopoietic cells and is required for hematopoietic cell proliferation. A 18-mer antisense ODN targeted to codons 2-7 of c-myb strongly inhibited or completely abolished clonogenic growth of a T-cell leukemia line (CCRF-CEM), as well as 78% of primary acute myelogenous leukemia cases examined, and 4 of

5 primary chronic myelogenous leukemia (CML) cases in blast crisis (Calabretta B et al.; Proc Natl Acd Sci USA 88:2351-2355, 1991).

Purging of bone marrow is used as a component in the treatment of several neoplasms, including acute and chronic leukemias. At present, marrow is cleansed of leukemic cells by a variety of agents such as immunologic reagents and chemotherapeutic drugs. Virosome encapsulated ODN targeted against one oncogene that confers a growth advantage to leukemic cells will prove therapeutically useful and, most important, more selective than conventional chemotherapeutic agents in eliminating leukemic cells while sparing normal progenitor cells.

Antisense-c-myb virosomes

Sense and antisense OPT corresponding to c-myb codons 2-9 were prepared. The sense and antisense c-myb sequences were 5'-GCCCGAAGACCCCGGCAC-3' and 5'-TGTGCCGGGGTCTTCGGGC-3', respectively. Encapsulation of OPT into DOTAP-virosomes was performed by the same method used for L-myc DOTAP virosomes. The human myeloid leukemia cell line KG-1 and the human acute lymphoblastic leukemia cell line CEM-C3 were exposed to sense and antisense c-myb virosomes. Proliferation of KG-1 cells is dependent on the protooncogene myb gene product, whereas CEM-C3 cells are not dependent on the product of c-myb gene. KG-1 cells were incubated with 25, 50, and 100 µl of sense and antisense c-myb virosomes containing 18, 36, and 72 pmol of sense and antisense OPT, respectively. The number of cells was determined at days 2, 3 and 4.

Addition of 25 µl of sense and antisense c-myb virosomes had only marginal effects on the cell growth (Fig.15). However, addition of 50 µl (Fig. 16) and 100 µl (Fig.17) strongly inhibited the cell growth. Higher doses of sense c-myb virosomes also showed inhibitory effects. It is assumed that these effects were not elicited by the virosomal membrane, because CEM-C3 cells were not influenced by the same virosome preparations (Fig.18).

Abbreviations used in the description

2'-OMe	2'-O methyl
CALLA	common acute lymphoblastic leukemia antigen
CAT	chloramphenicol acetyltransferase
DOTAP	N-[(1,2,3-dioleoyloxy)-propyl]-N,N,N-trimethylammoniummethyl-sulfate
DOTMA	N-[(1,2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride
FITC-OPT	fluorescein isothiocyanate-labeled oligodeoxyribonucleotide phosphorothioate
G418	Geneticin® disulfat (antibiotic G418)
HA	hemagglutinin
IL-6	Interleukin 6
MPB.PE	N-[4-(p-maleimido)-phenylbutyryl]-phosphatidylethanolamine (= a crosslinker-phospholipid complex)
msc	mixed sequence control
NA	neuraminidase
Octyl-POE	n-octyl-oligo-oxyethylene
ODN	oligodeoxynucleotides
OEG	octaethyleneglycol monododecylether ($C_{12}E_8$)
OPT	oligodeoxyribonucleotide phosphorothioate(s)
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PNA	peptide nucleic acid
SCLC	small cell lung cancer
SV40	Simian virus 40

CLAIMS

1. A lipid vesicle with a positively charged lipid bilayer membrane comprising cationic and/or polycationic lipids and at least one natural or synthetic viral fusion peptide integrated in or covalently linked to the membrane.

2. The vesicle according to claim 1, whereinthe membrane comprises - based on total lipids -

(i) 100 % by weight of cationic and/or polycationic lipids; or

(ii) 90 to 95 % by weight of cationic and/or polycationic lipids and 5 to 10 % by weight of phosphatidylethanolamine; or

(iii) 45 to 90 % by weight of cationic and/or polycationic lipids, 5 to 10 % by weight of phosphatidylethanolamine and 5 to 50 % by weight of phosphatidylcholine.

3. The vesicle according to claim 1 or 2, wherein

(i) the cationic lipids comprise at least one member selected from the group consisting of N-[1,2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA); N-[1,2,3-dioleoyloxy)-propyl]-N,N,N-trimethylammonium-methylsulfate (DOTAP); N-t-butyl-N'-tetradecyl-3-tetradecylaminopropionamidine; and

(ii) the polycationic lipids comprise at least one member selected from the group consisting of 1,3-dipalmitoyl-2-phosphatidylethanolamidospermine (DPPES); dioctadecylamidoglycylspermine (DOGS); 2,3-dioleyloxy-N-[2(spermine-carboxamido)ethyl]-N,N-dimethyl-1-propaneaminiumtrifluoroacetate (DOSPA); 1,3-dioleoyloxy-2-(6-carboxy-spermyl)-propylamide (DOSPER); and N,N,N',N'-tetramethyl-N,N'-bis(2-hydroxyethyl)-2,3-dioleoyloxy-1,4-butanediammoniumiodide (THDOB).

4. The vesicle according to one or more of the preceding claims, wherein the fusion peptide is selected from the group consisting of a hemagglutinin trimer or monomer, one or both cleaved subunits thereof, glycopeptides HA1 and HA2, fusion peptide isolated from natural sources, and synthetic fusion peptide.

5. The vesicle according to one or more of the preceding claims, wherein the membrane further comprises at least one cell-specific marker and at least one bifunctional crosslinking agent and wherein said cell-specific marker is linked to said membrane by means of said crosslinking agent.

6. The vesicle according to claim 5, wherein said cell-specific marker is selected from the group consisting of a monoclonal antibody, a $F(ab')_2$ fragment, a Fab' fragment, a cytokine and a growth factor.

7. The vesicle according to one or more of the preceding claims, further comprising desired genetic material entrapped in said vesicle, preferably selected from the group consisting of short chain DNA or RNA, deoxyribonucleotides, oligodeoxyribonucleotides, oligodeoxyribonucleotide selenoates, oligodeoxyribonucleotide phosphorothioates, oligodeoxyribonucleotide phosphoramidates, oligodeoxyribonucleotide methylphosphonates, peptide nucleic acids, ribonucleotides, oligoribonucleotides, oligoribonucleotide phosphorothioates, 2'-OMe-oligoribonucleotide phosphates, 2'-OMe-oligoribonucleotide phosphorothioates, ribozymes, genes, plasmids and vectors.

8. The vesicle according to one or more of the preceding claims, whereinits diameter is in the range of 120 - 180 nm.

9. A process for the manufacture of a vesicle with a positively charged lipid bilayer membrane and at least one viral fusion peptide, wherein

a) at least one natural or synthetic viral fusion peptide is dissolved in a suitable buffer containing a non-ionic detergent;
b) cationic and/or polycationic lipids are added to the solution and dissolved; and
c) the detergent is removed by treating the solution with polystyrene microcarrier beads, preferably Biobeads SM-2, resulting in the formation of a suspension containing said positively charged lipid bilayer vesicles.

10. The process according to claim 9, wherein

(i) the cationic lipids comprise at least one member selected from the group consisting of

N-[1,2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA); N-[1,2,3-dioleoyloxy)-propyl]-N,N,N-trimethylammonium-methylsulfate (DOTAP); N-t-butyl-N´-tetradecyl-3-tetradecylaminopropionamidine; and

(ii) the polycationic lipids comprise at least one member selected from the group consisting of
1,3-dipalmitoyl-2-phosphatidylethanolamidospermine (DPPES); dioctadecylamidoglycylspermine (DOGS); 2,3-dioleoyloxy-N-[2(spermine-carboxamido)ethyl]-N,N-dimethyl-1-propaneaminiumtrifluoroacetate (DOSPA); 1,3-dioleoyloxy-2-(6-carboxy-spermyl)-propylamide (DOSPER); and N,N,N´,N´-tetramethyl-N,N´-bis(2-hydroxyethyl)-2,3-dioleoyloxy-1,4-butanediammoniumiodide (THDOB).

11. The process according to claim 9 or 10, wherein said buffer is a HEPES buffer and further comprises 10 to 250 µmol per ml of said non-ionic detergent, preferably selected from the group consisting of octaethyleneglycol monododecylether and n-octyl-oligo-oxyethylene.

12. The process according to one or more of claims 9 to 11, wherein said viral fusion peptide is selected from the group consisting of a hemagglutinin trimer or monomer, one or both cleaved subunits thereof, glycopeptides HA1 and HA2, fusion peptide isolated from natural sources, and synthetic fusion peptide.

13. The process according to one or more of claims 9 to 12, wherein phosphatidylcholine and/or phosphatidylethanolamine is admixed to the solution of step (b).

14. The process according to one or more of claims 9 to 13, wherein further at least one cell-specific marker and a bifunctional crosslinking agent are added to the solution of step (b), preferably in the form of a pre-formed molecule complex consisting of phosphatidylethanolamine, bifunctional crosslinking agent and cell-specific marker.

15. The process according to one or more of claims 9 to 14, wherein step (b) comprises the addition of - based on total lipids - either
(i) 100 % by weight of cationic and/or polycationic lipids; or

(ii) 90 to 95 % by weight of cationic and/or polycationic lipids and 5 to 10 % by weight of phosphatidylethanolamine; or

(iii) 45 to 90 % by weight of cationic and/or polycationic lipids, 5 to 10 % by weight of phosphatidylethanolamine and 5 to 50 % by weight of phosphatidylcholine.

16. The process according to one or more of claims 9 to 15, wherein said microcarrier beads have a wet mesh size of 20 -50 (0.84 - 0.30 mm). and the solution in step (c) is treated up to four times with said microcarrier beads.

17. The process according to one or more of claims 9 to 16, wherein a desired genetic material is added to the solution of step (b).

18. The process according to one or more of claims 9 to 16, wherein a desired genetic material is added to the vesicle suspension of step (c), whereafter the resulting mixture is subjected to ultrasonication to incorporate said desired genetic material into said vesicles and whereupon non-incorporated material is separated from the vesicles, preferably by gel filtration.

19. The process according to one or more of claims 14 to 18, wherein said cell-specific marker is selected from the group consisting of a monoclonal antibody, $F(ab')_2$ fragment, Fab' fragment, a cytokine and a growth factor.

20. The process according to one or more of claims 14 to 19, wherein said crosslinking agent is a heterobifunctional succinimidyl derivative, preferably selected from the group consisting of bis-N-succinimidyl derivatives and photoactivatable heterobifunctional cross-linking agents.

21. The process according to one or more of claims 14 to 20, wherein said preformed molecule complex is obtained from a reaction mixture wherein said cell specific marker is bound via a thiol group to the crosslinking portion of a covalently linked crosslinker-phosphatidylethanol-amine molecule preferably selected from the group consisting of

N-[4-(p-maleimidophenyl)-butyryl]-phosphatidylethanolamine (MPB.PE) and 4-(N-maleimidomethyl)cyclohexane-1-carboxylate-phosphatidylethanolamine (MCC.PE).

22. The process according to claim 21, wherein unreacted crosslinker-phosphatidylethanolamine molecules, particularly unreacted MPB.PE or MCC.PE, are removed from the reaction mixture by gel chromatography, preferably by affinity chromatography with an agarose matrix, most preferably by reduced Thiopropylsepharose 6B.

23. The process according to one or more of claims 9 to 22, wherein said desired genetic material is selected from the group consisting of short chain DNA or RNA, deoxyribonucleotides, oligodeoxyribonucleotides, oligodeoxyribonucleotide selenoates, oligodeoxyribonucleotide phosphorothioates, oligodeoxyribonucleotide phosphoramidates, oligodeoxyribonucleotide methylphosphonates, peptide nucleic acids, ribonucleotides, oligoribonucleotides, oligoribonucleotide phosphorothioates, 2'-OMe-oligoribonucleotide phosphates, 2'-OMe-oligoribonucleotide phosphorothioates, ribozymes, genes, plasmids and vectors.

24. A positively charged lipid vesicle as defined in anyone of claims 1 to 8 and/or obtainable by a process as defined in anyone of claims 9 to 23 for specific or unspecific delivery of genetic material to target cells or tissues.

25. A positively charged lipid vesicle as defined in anyone of claims 1 to 8 and/or obtainable by a process as defined in anyone of claims 9 to 23 for use as a medicament, preferably for the prophylactic and/or therapeutic treatment of humans or animals.

26. Use of a lipid vesicle with a positively charged lipid bilayer membrane, the vesicle comprising cationic and/or polycationic lipids, at least one natural or synthetic viral fusion peptide integrated in or covalently linked to the membrane, and preferably at least one cell-specific marker linked to the membrane, as a carrier system for drug delivery.

27. Use according to claim 26, for non-infectious transfer of genetic material to resting or proliferating mammalian cells.

28. Use of a lipid vesicle with a positively charged lipid bilayer membrane, the vesicle comprising cationic and/or polycationic lipids, at least one natural or synthetic viral fusion peptide integrated in or covalently linked to the membrane, and preferably at least one cell-specific marker linked to the membrane, for the manufacture of a pharmaceutical composition for the treatment of cancer.

29. Use according to anyone of claims 26 to 28, wherein the fusion peptide is selected from the group consisting of hemagglutinin trimer or monomer, one or both cleaved subunits thereof, glycopeptides HA1 and HA2, fusion peptide isolated from natural sources, and synthetic fusion peptide.

30. Use according to anyone of claims 26 to 29, wherein the cell-specific marker is selected from the group consisting of a monoclonal antibody, a $F(ab')_2$ fragment, a Fab' fragment, a cytokine and a growth factor.

31. Use according to anyone of claims 26 to 30, wherein the vesicle further contains desired genetic material, preferably selected from the group consisting of short chain DNA or RNA, deoxyribonucleotides, oligodeoxyribonucleotides, oligodeoxyribonucleotide selenoates, oligodeoxyribonucleotide phosphorothioates, oligodeoxyribonucleotide phosphoramidates, oligodeoxyribonucleotide methylphosphonates, peptide nucleic acids, ribonucleotides, oligoribonucleotides, oligoribonucleotide phosphorothioates, 2'-OMe-oligoribonucleotide phosphates, 2'-OMe-oligoribonucleotide phosphorothioates, ribozymes, genes, plasmids and vectors.

32. Use according to anyone of claims 26 to 31, wherein the vesicle comprises at least one antisense oligonucleotide suitable for antisense therapy of cancer, leukemias, and viral infections.

33. Use according to claim 32, wherein the antisense oligonucleotide is targeting protooncogene or oncogene encoded mRNA.

34. Use of a positively charged vesicle as defined in anyone of claims 1 to 8 and/or obtainable by a process as defined in anyone of claims 9 to 23 for the manufacture of a pharmaceutical composition useful for diagnostic application and/or for the prophylactic and/or therapeutic treatment of humans or animals.

FIG. 1

Fig. 2

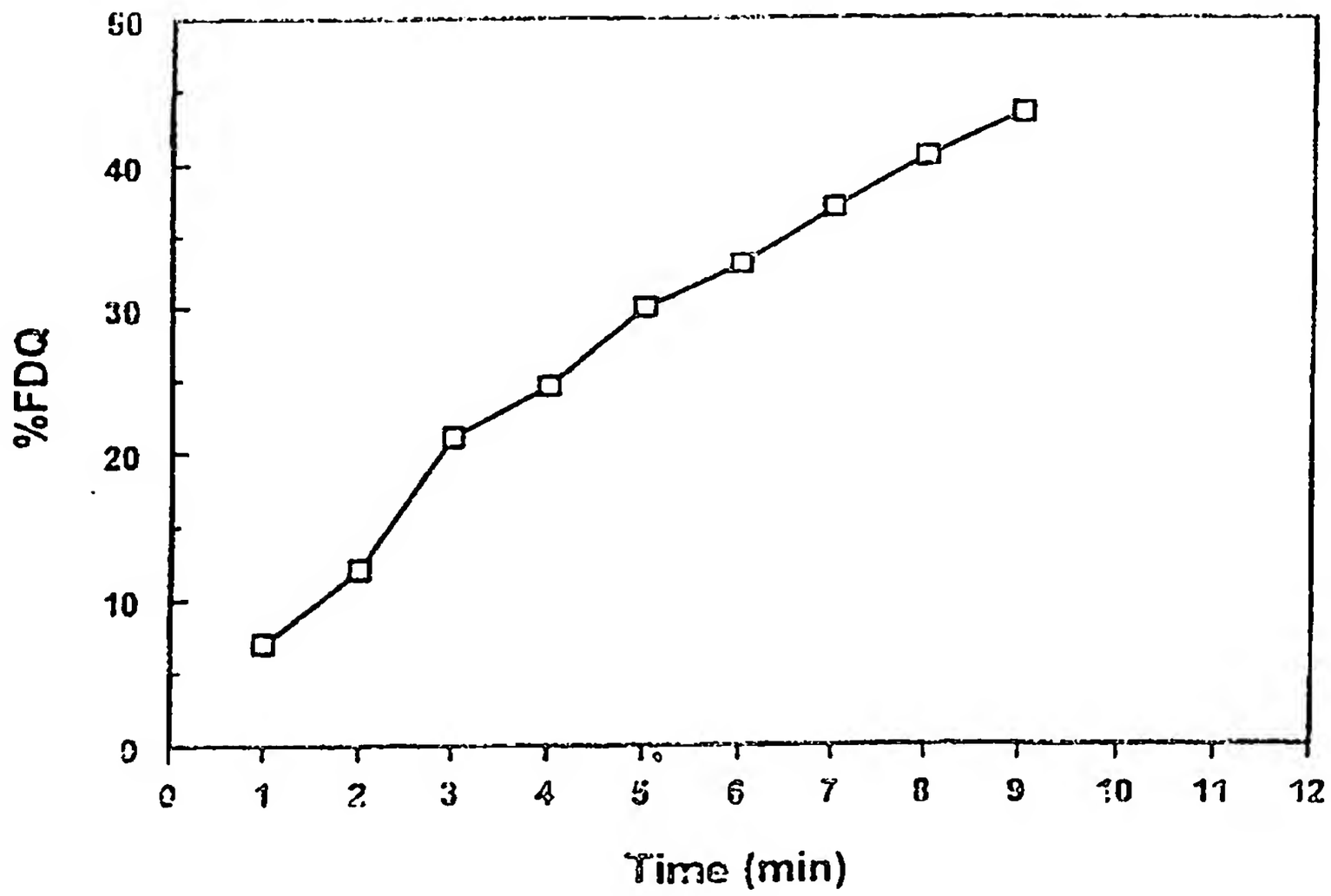


FIG. 3

Fig.4

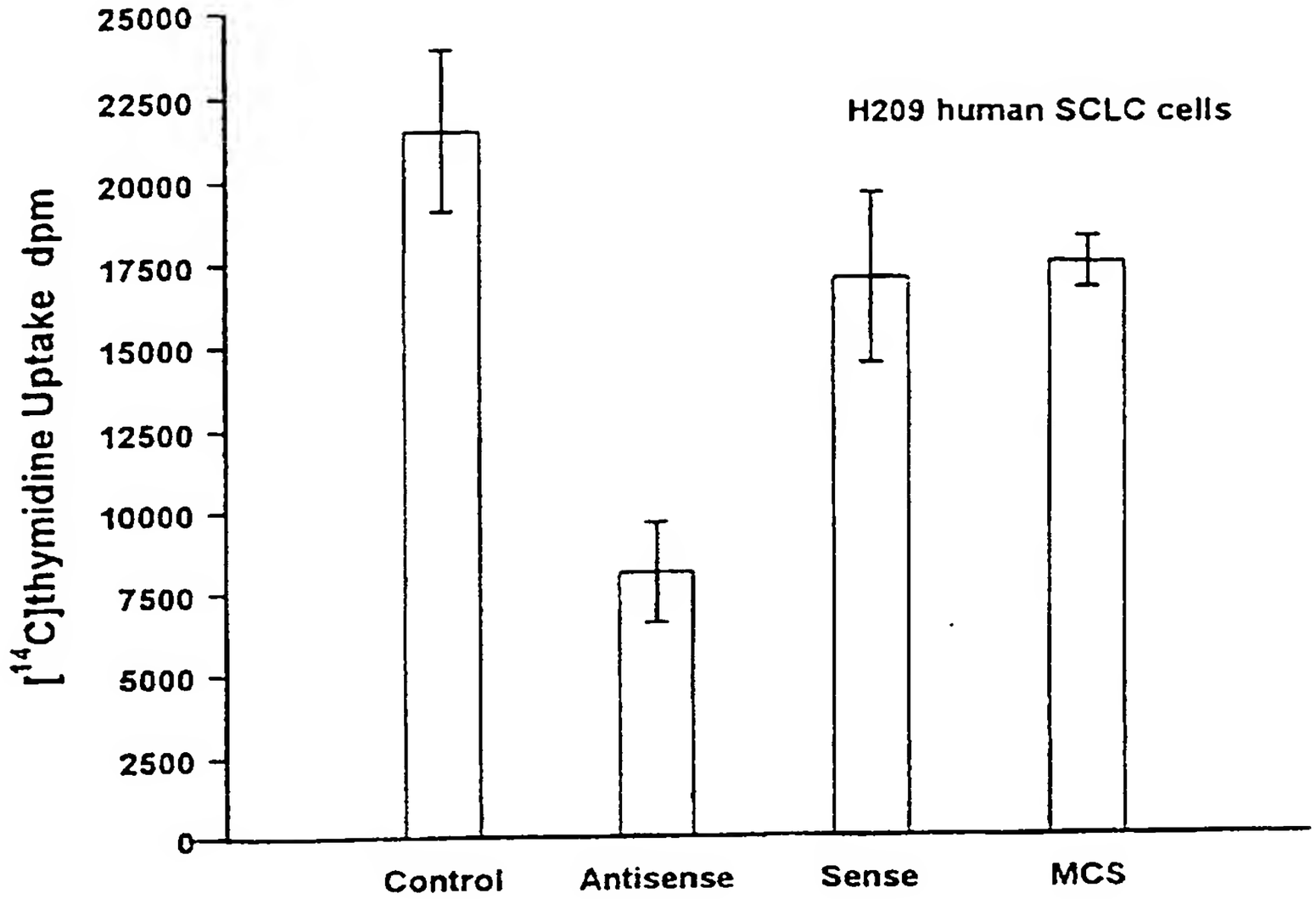
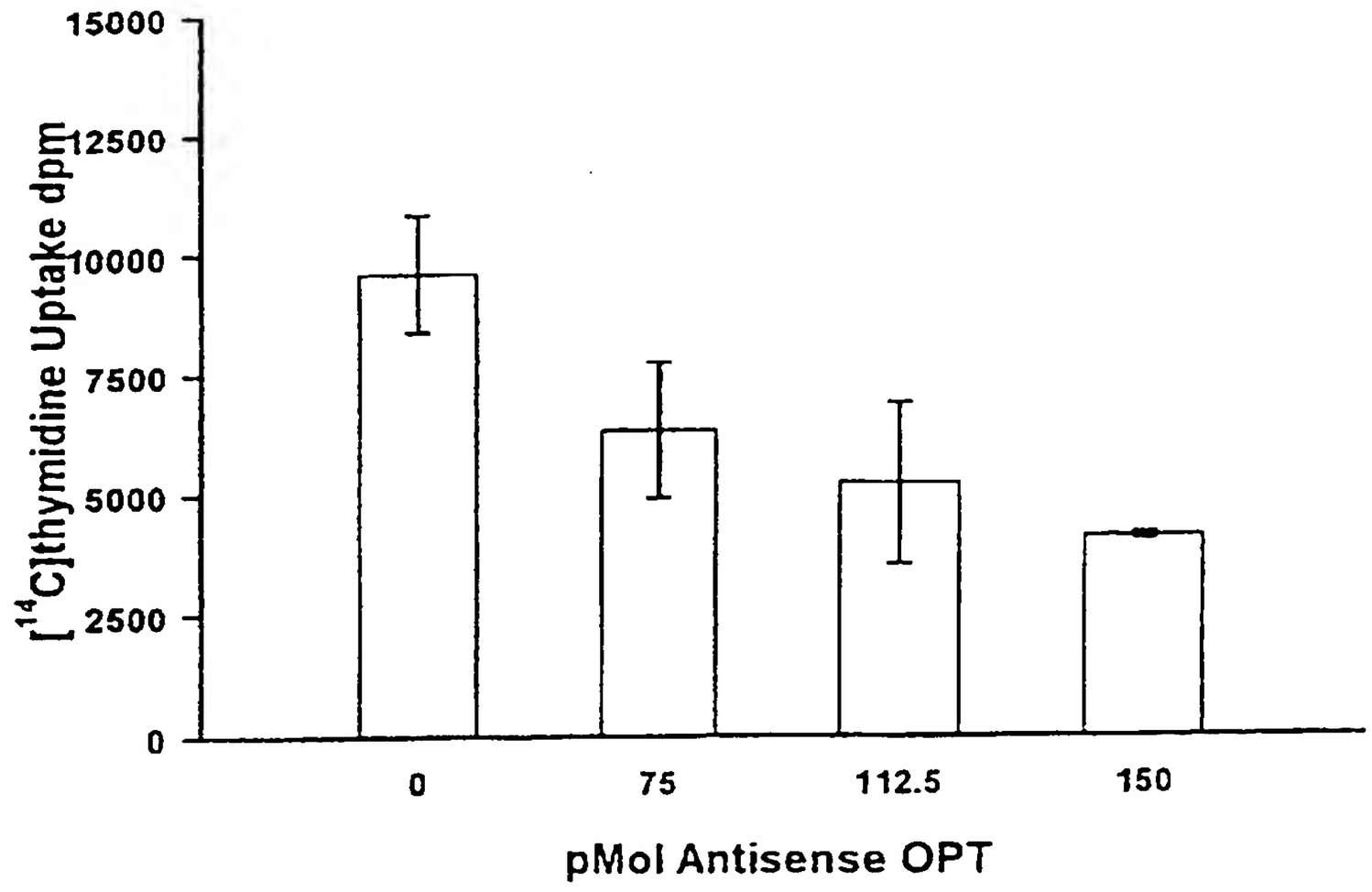

H209 human SCLC cells
[14C]thymidine Uptake dpm
25000
22500
20000
17500
15000
12500
10000
7500
5000
2500
0
Control
Antisense
Sense
MCS

Fig.5


[14C]thymidine Uptake dpm
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12500
10000
7500
5000
2500
0
0
75
112.5
150
pMol Antisense OPT

Fig.6

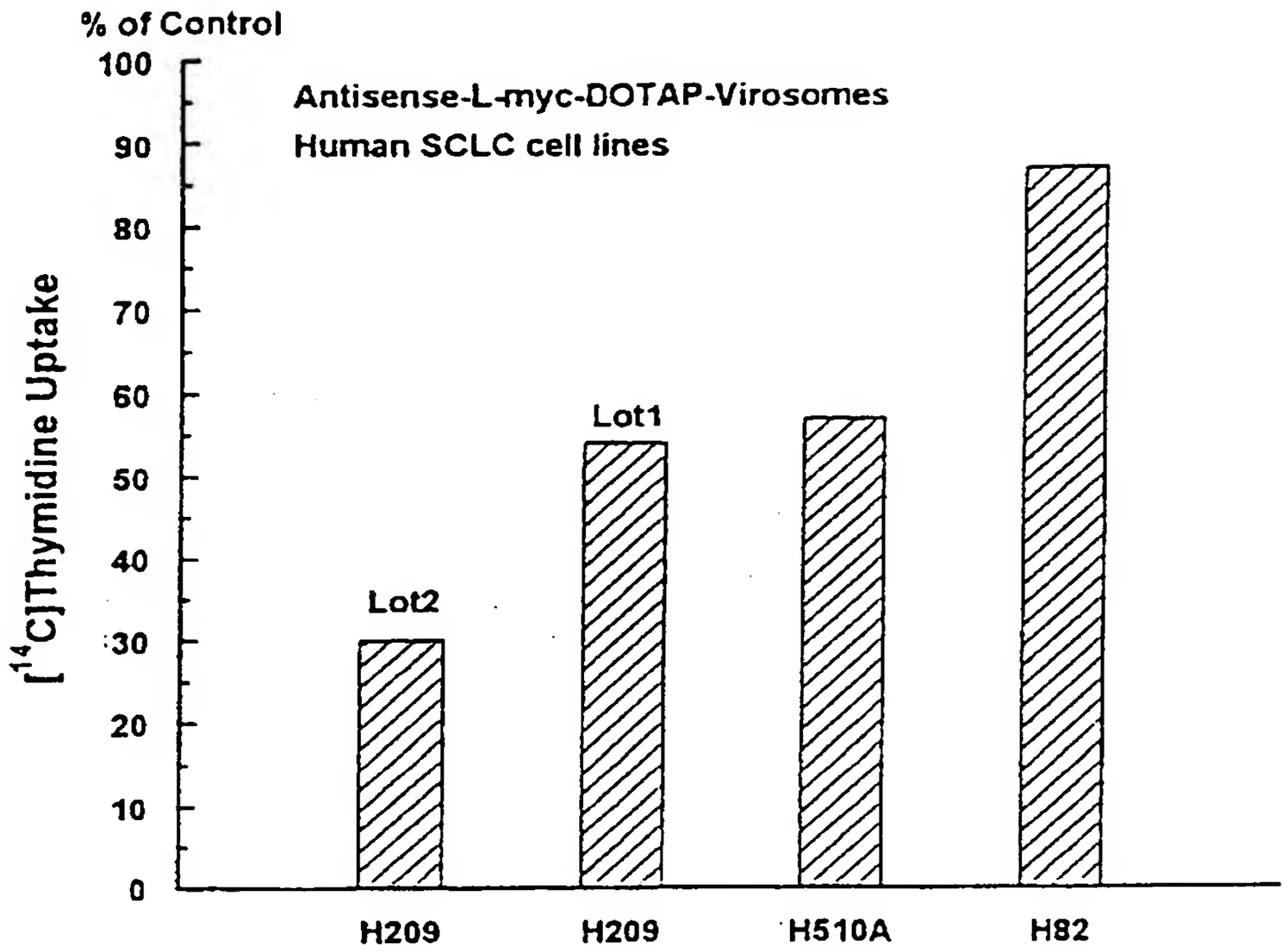

% of Control
100
90
80
70
60
50
40
30
20
10
0
[14C]Thymidine Uptake
Antisense-L-myc-DOTAP-Virosomes
Human SCLC cell lines
Lot2
Lot1
H209
H209
H510A
H82

Fig.7a

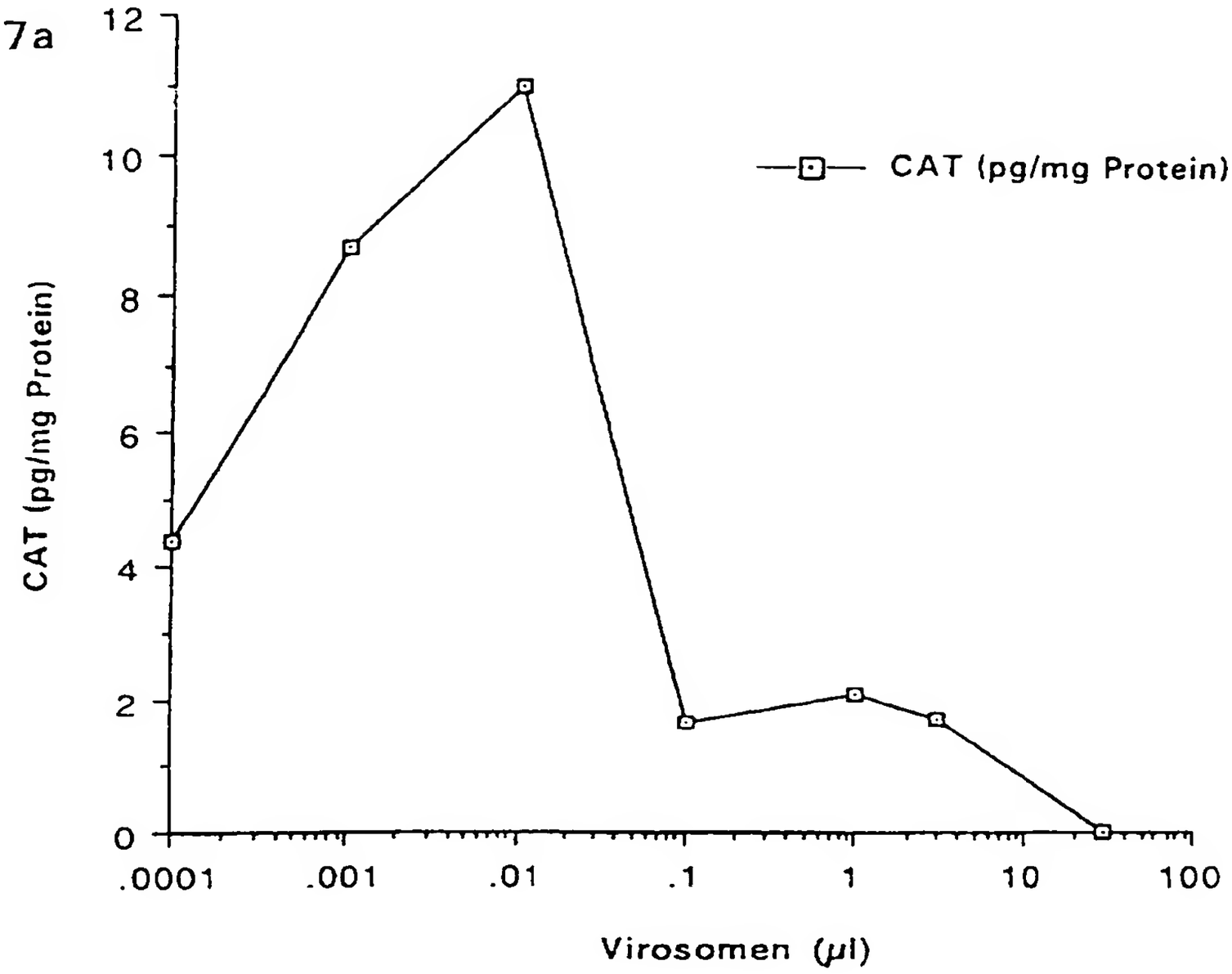
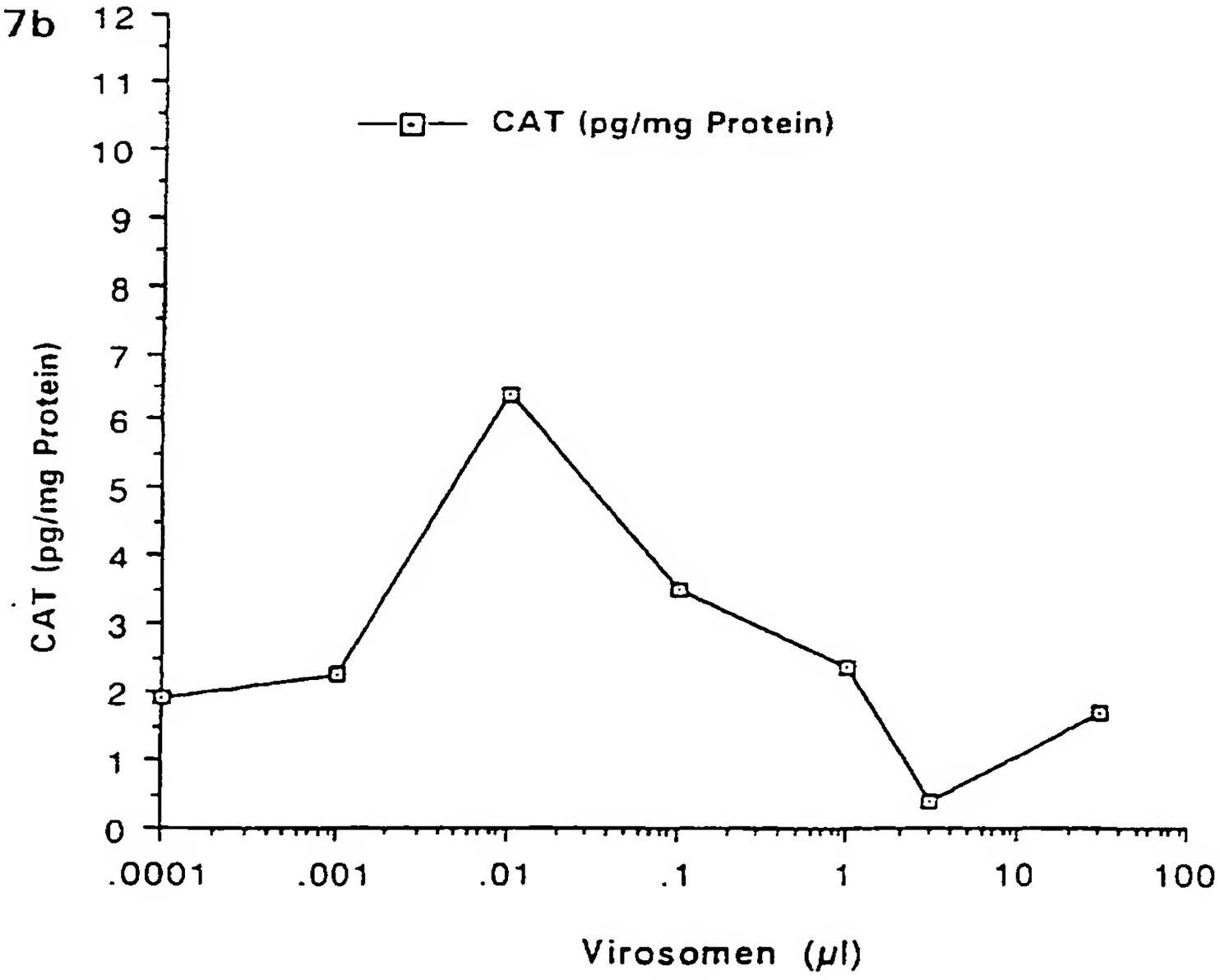

12
10
8
6
4
2
0
CAT (pg/mg Protein)
CAT (pg/mg Protein)
.0001
.001
.01
.1
1
10
100
Virosomen (µl)

Fig.7b


12
11
10
9
8
7
6
5
4
3
2
1
0
CAT (pg/mg Protein)
CAT (pg/mg Protein)
.0001
.001
.01
.1
1
10
100
Virosomen (µl)

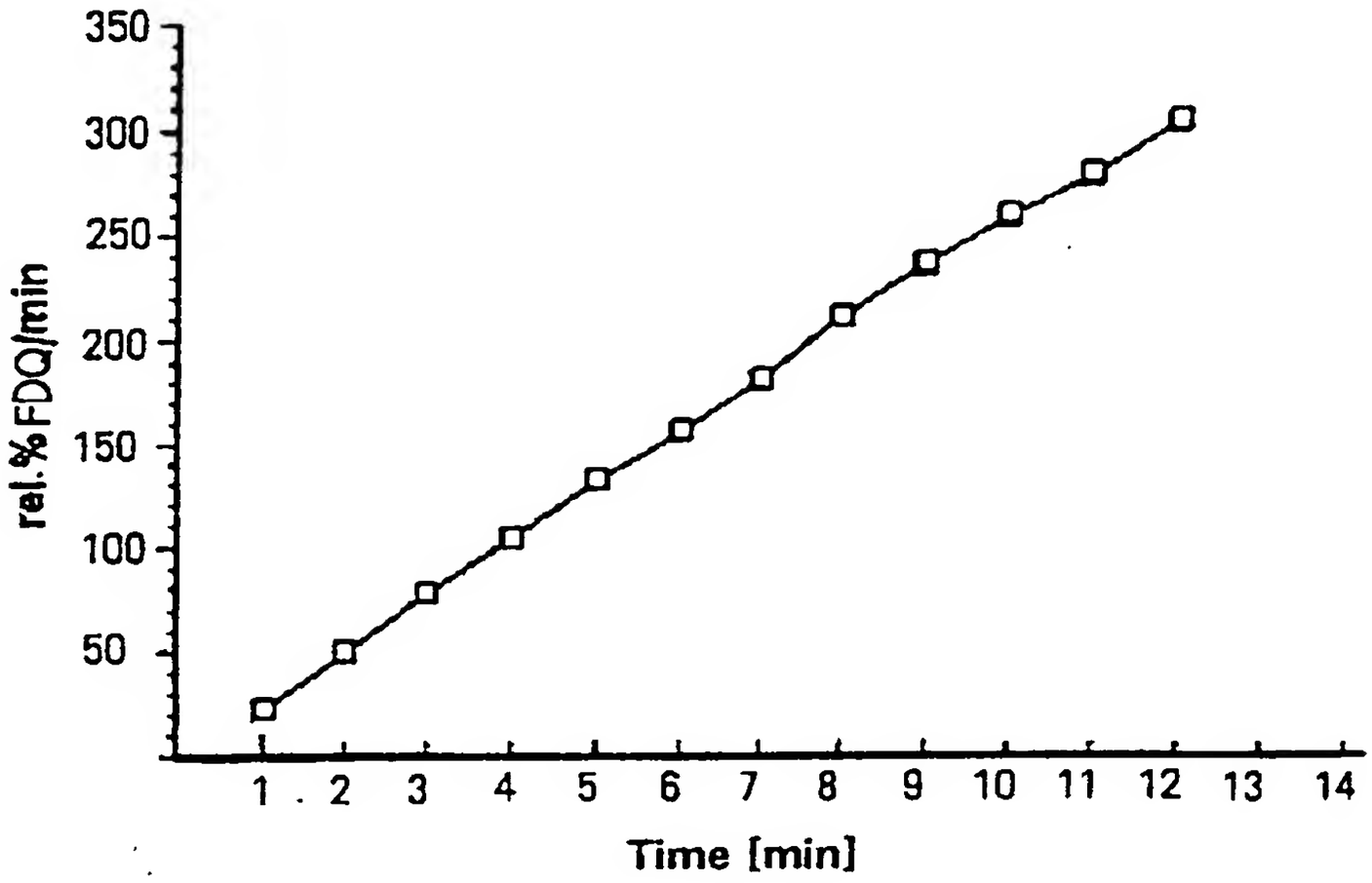

350
300
250
200
150
100
50
rel.%FDQ/min
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Time [min]

FIG. 8

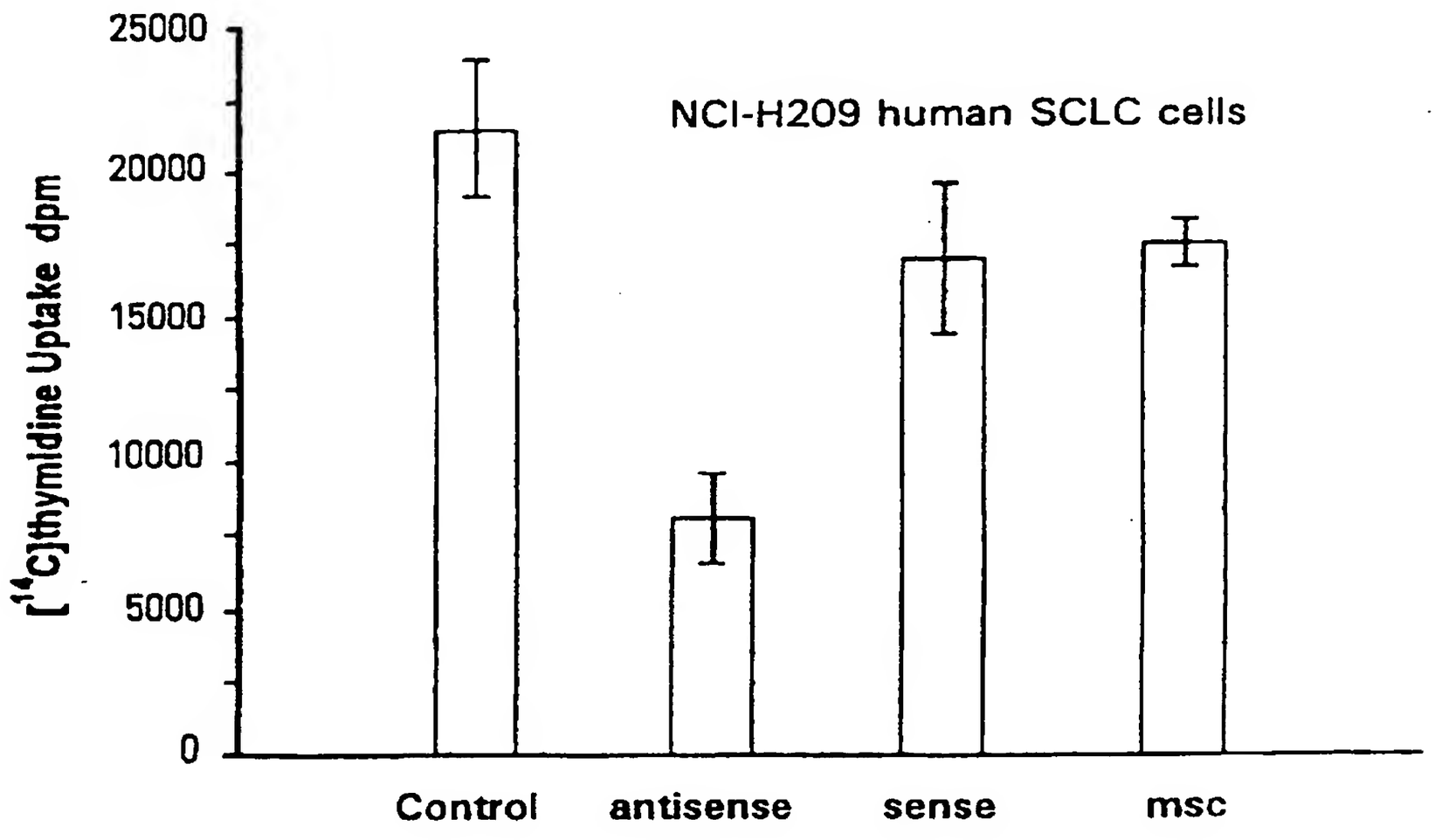

25000
20000
15000
10000
5000
0
[14C]thymidine Uptake dpm
NCI-H209 human SCLC cells
Control
antisense
sense
msc

FIG. 9

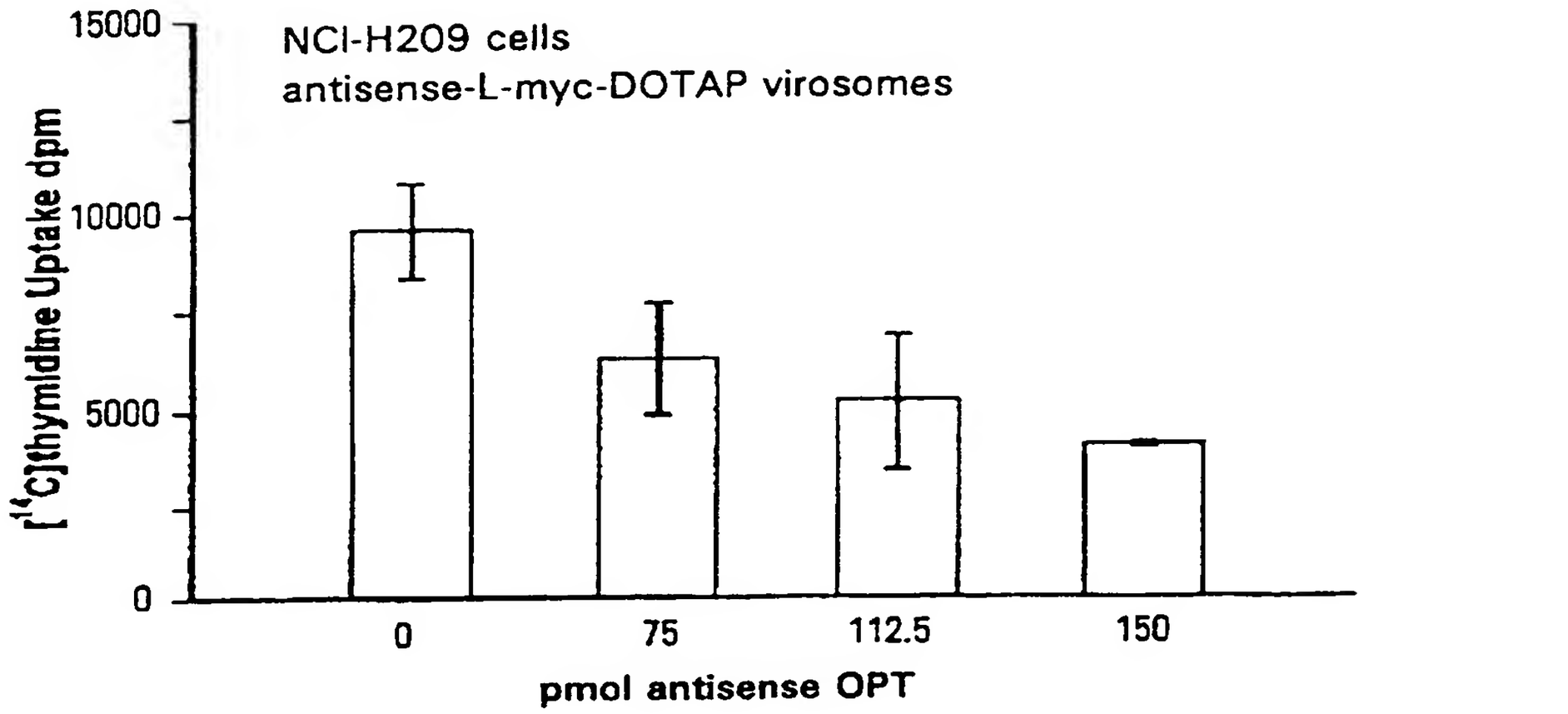

15000
10000
5000
0
[14C]thymidine Uptake dpm
NCI-H209 cells
antisense-L-myc-DOTAP virosomes
0
75
112.5
150
pmol antisense OPT

FIG. 10

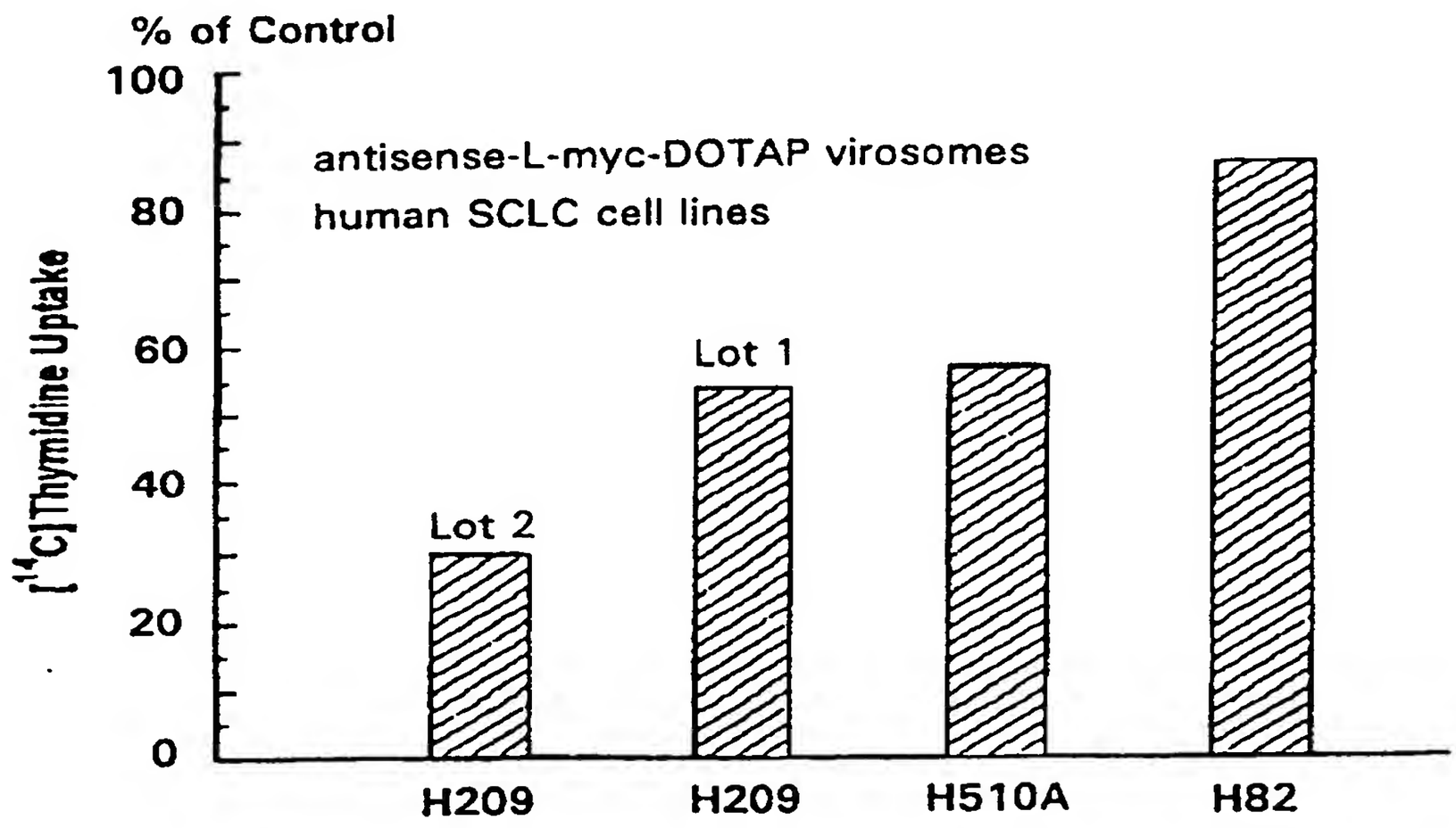

% of Control
100
80
60
40
20
0
[14C]Thymidine Uptake
antisense-L-myc-DOTAP virosomes
human SCLC cell lines
Lot 2
Lot 1
H209
H209
H510A
H82

FIG. 11

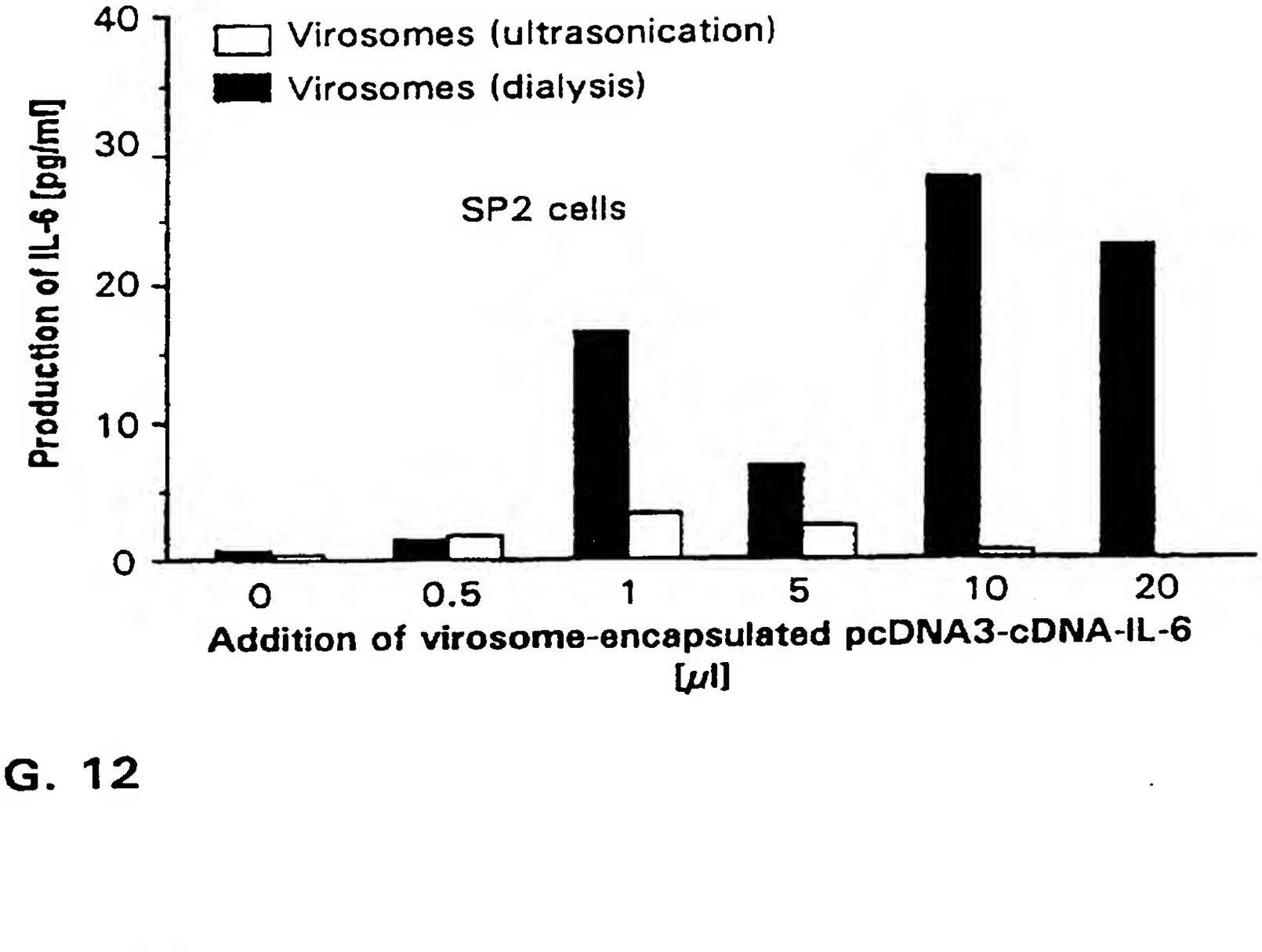

Virosomes (ultrasonication)
Virosomes (dialysis)
SP2 cells
Production of IL-6 [pg/ml]
40
30
20
10
0
0
0.5
1
5
10
20
Addition of virosome-encapsulated pcDNA3-cDNA-IL-6
[µl]

FIG. 12

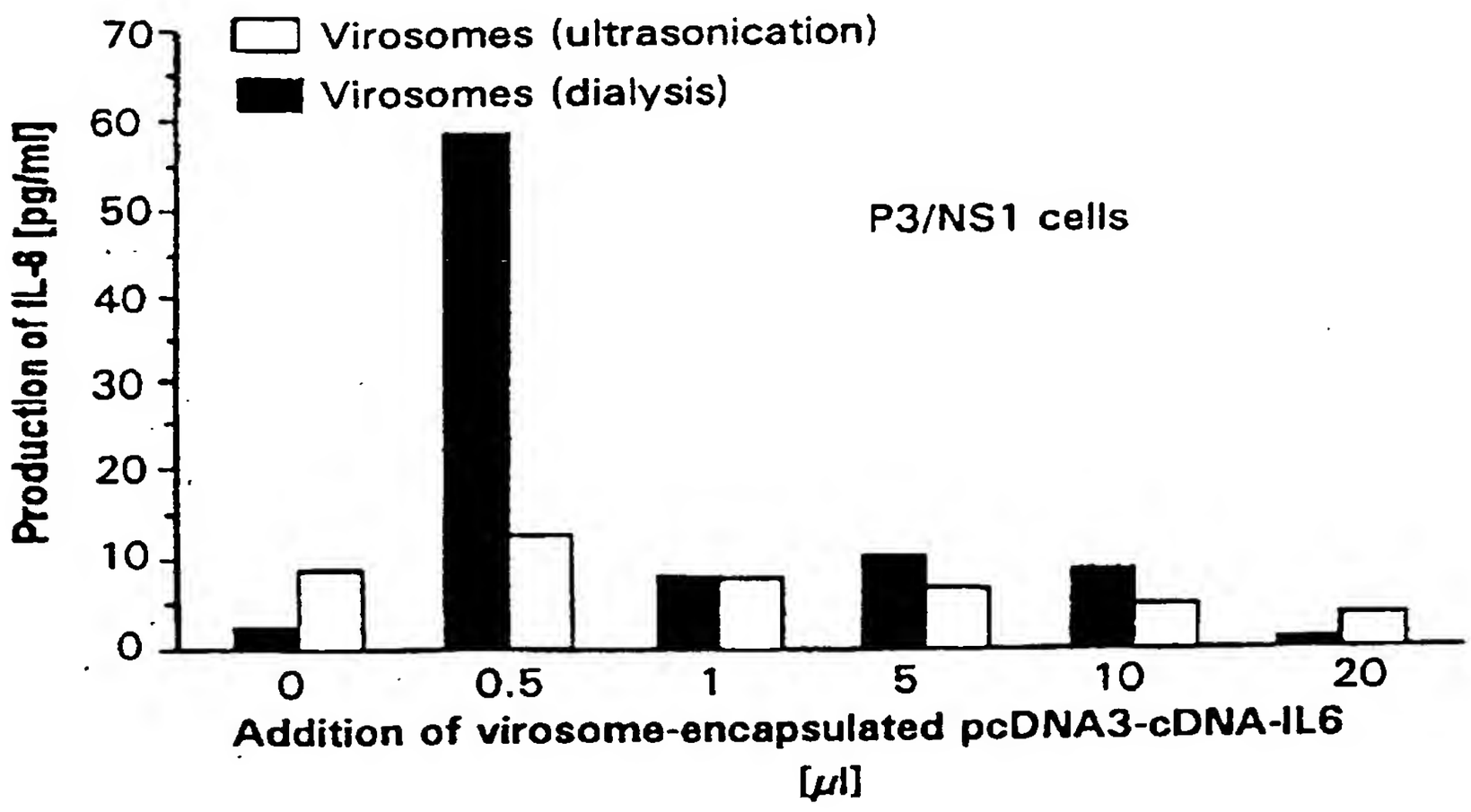

Virosomes (ultrasonication)
Virosomes (dialysis)
P3/NS1 cells
Production of IL-6 [pg/ml]
70
60
50
40
30
20
10
0
0
0.5
1
5
10
20
Addition of virosome-encapsulated pcDNA3-cDNA-IL6
[µl]

FIG. 13

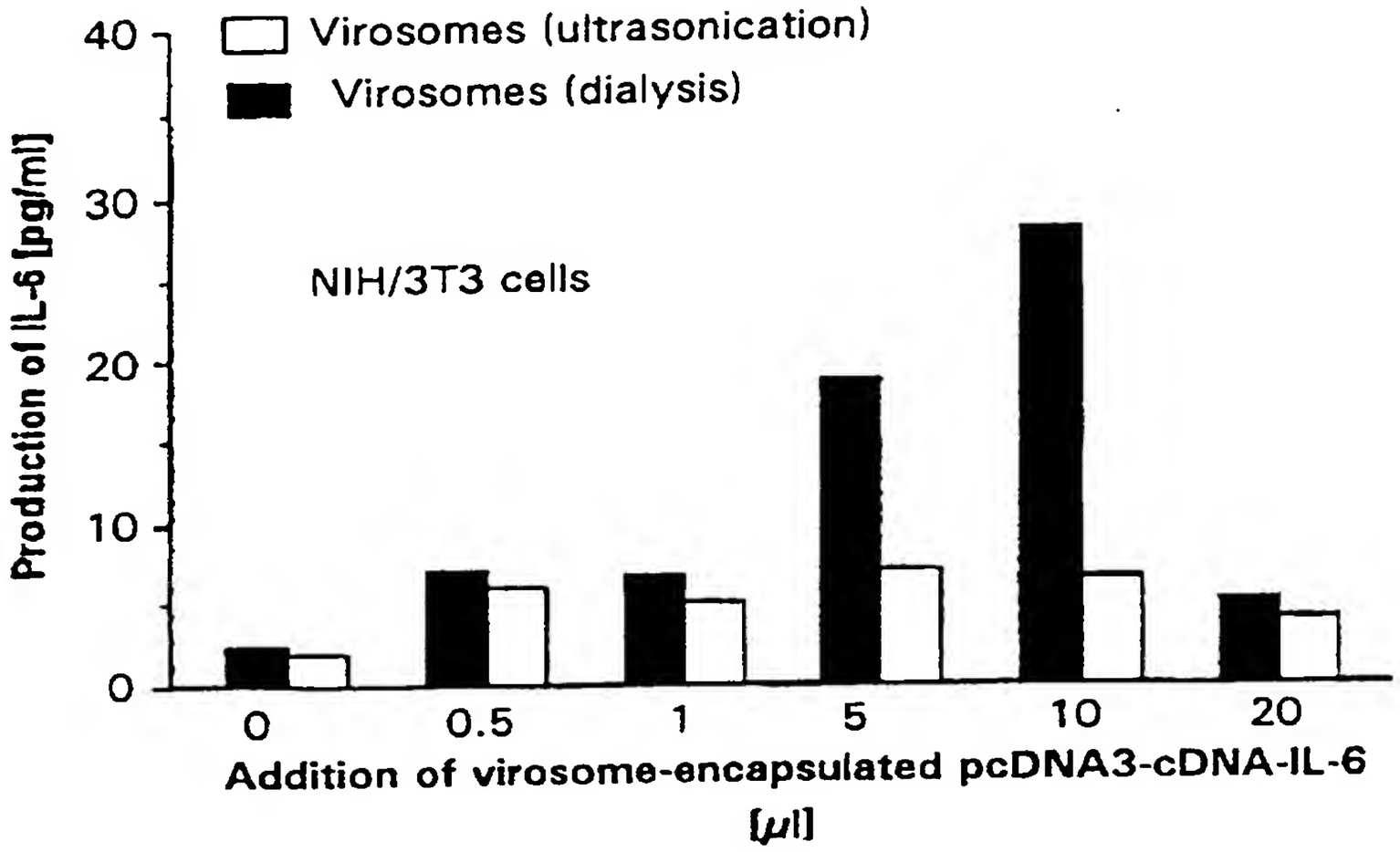

Virosomes (ultrasonication)
Virosomes (dialysis)
NIH/3T3 cells
Production of IL-6 [pg/ml]
40
30
20
10
0
0
0.5
1
5
10
20
Addition of virosome-encapsulated pcDNA3-cDNA-IL-6
[µl]

FIG. 14

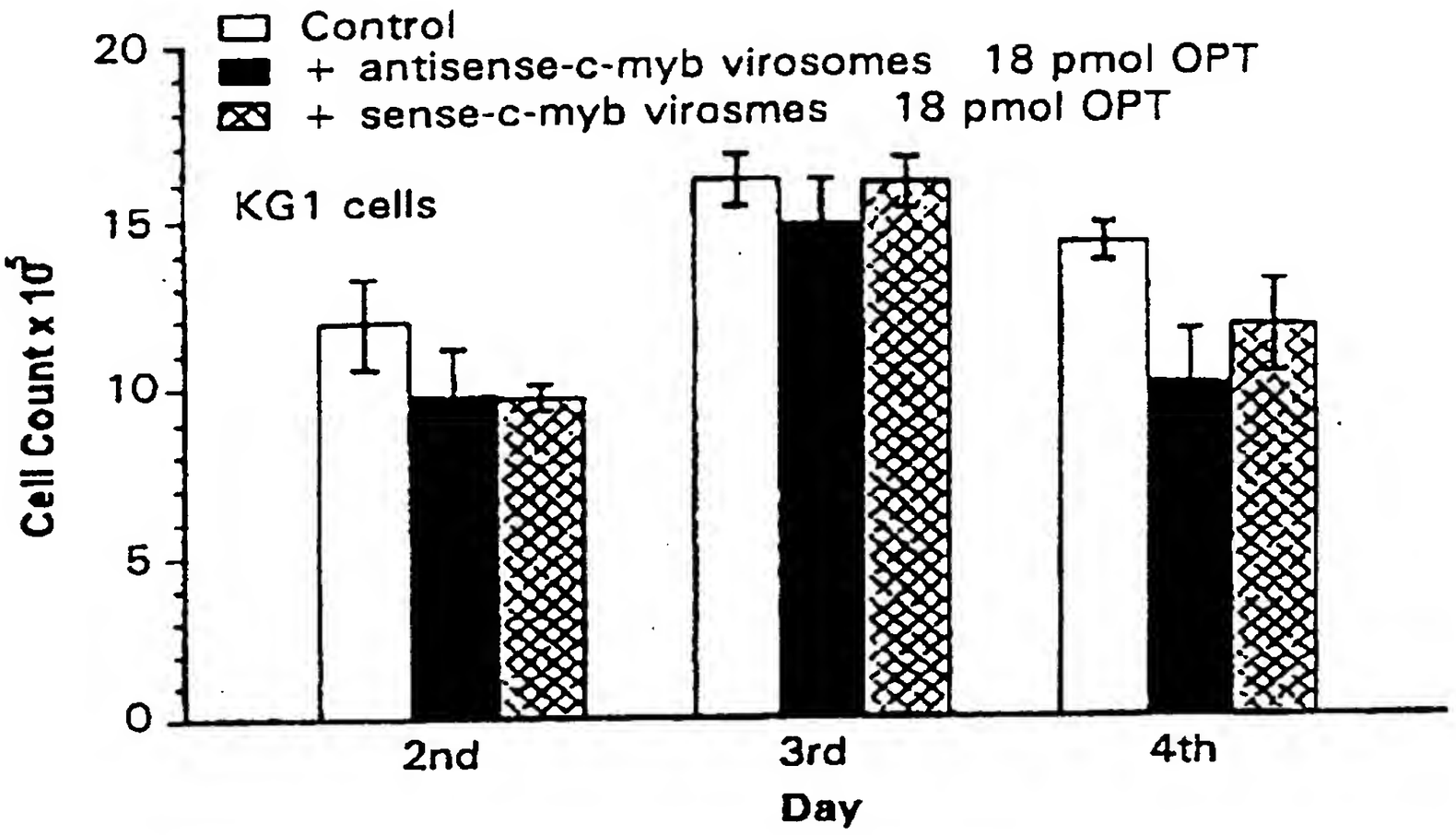

Control
+ antisense-c-myb virosomes 18 pmol OPT
+ sense-c-myb virosmes 18 pmol OPT
KG1 cells
Cell Count x 10³
20
15
10
5
0
2nd
3rd
4th
Day

FIG. 15

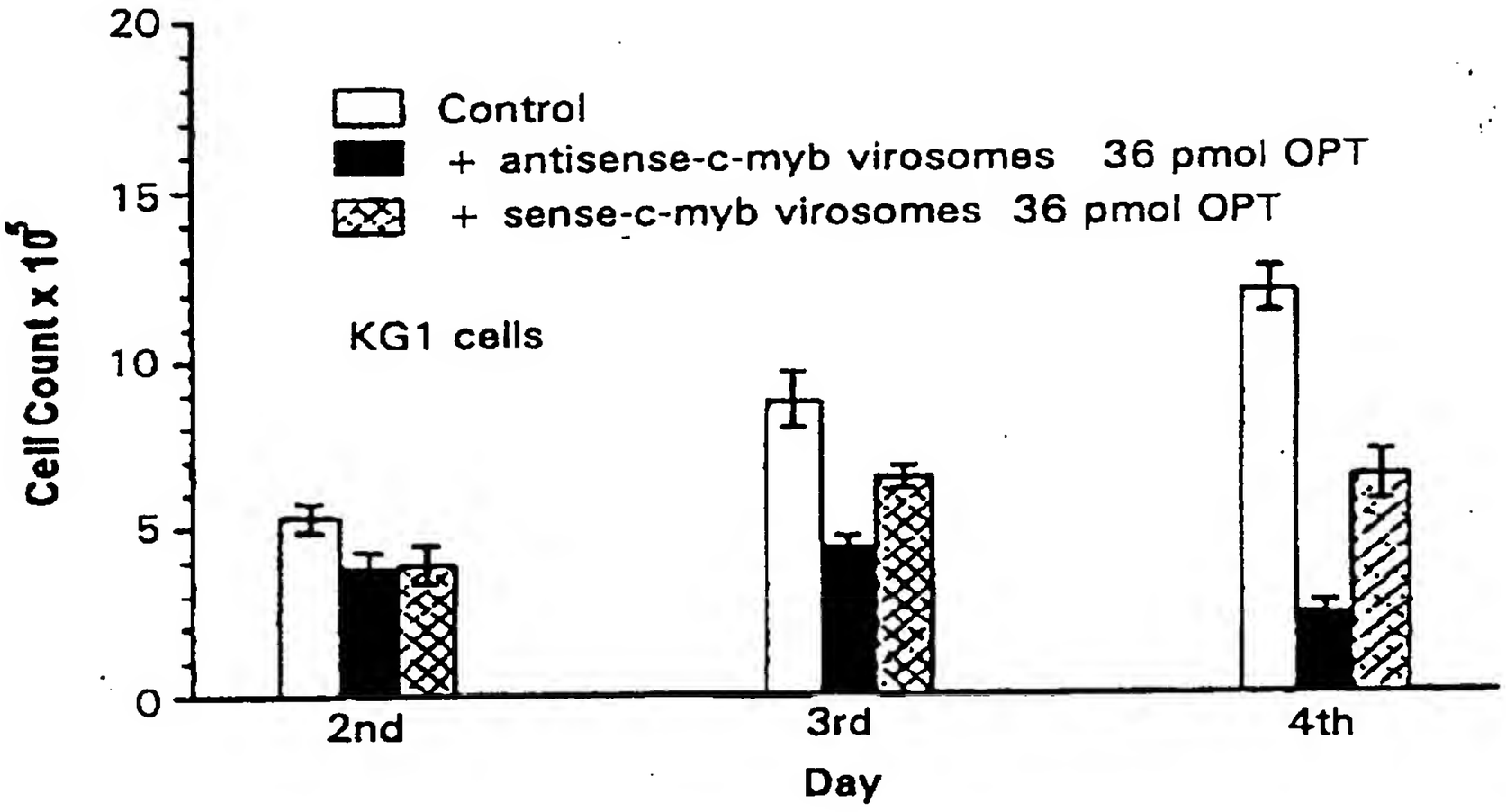

Control
+ antisense-c-myb virosomes 36 pmol OPT
+ sense-c-myb virosomes 36 pmol OPT
KG1 cells
Cell Count x 10³
20
15
10
5
0
2nd
3rd
4th
Day

FIG. 16

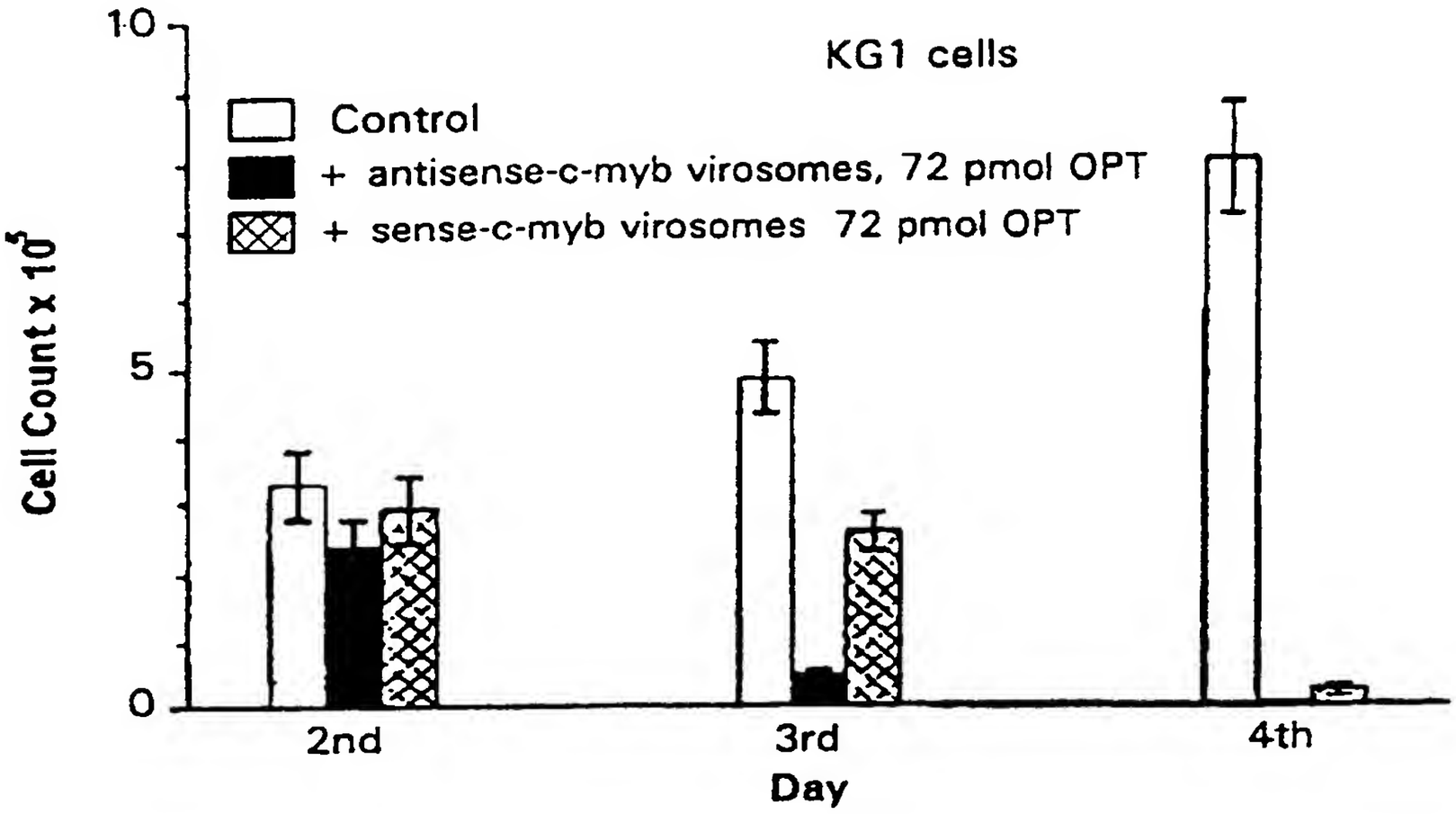

KG1 cells
Control
+ antisense-c-myb virosomes, 72 pmol OPT
+ sense-c-myb virosomes 72 pmol OPT
Cell Count x 10⁵
10
5
0
2nd
3rd
4th
Day

FIG. 17

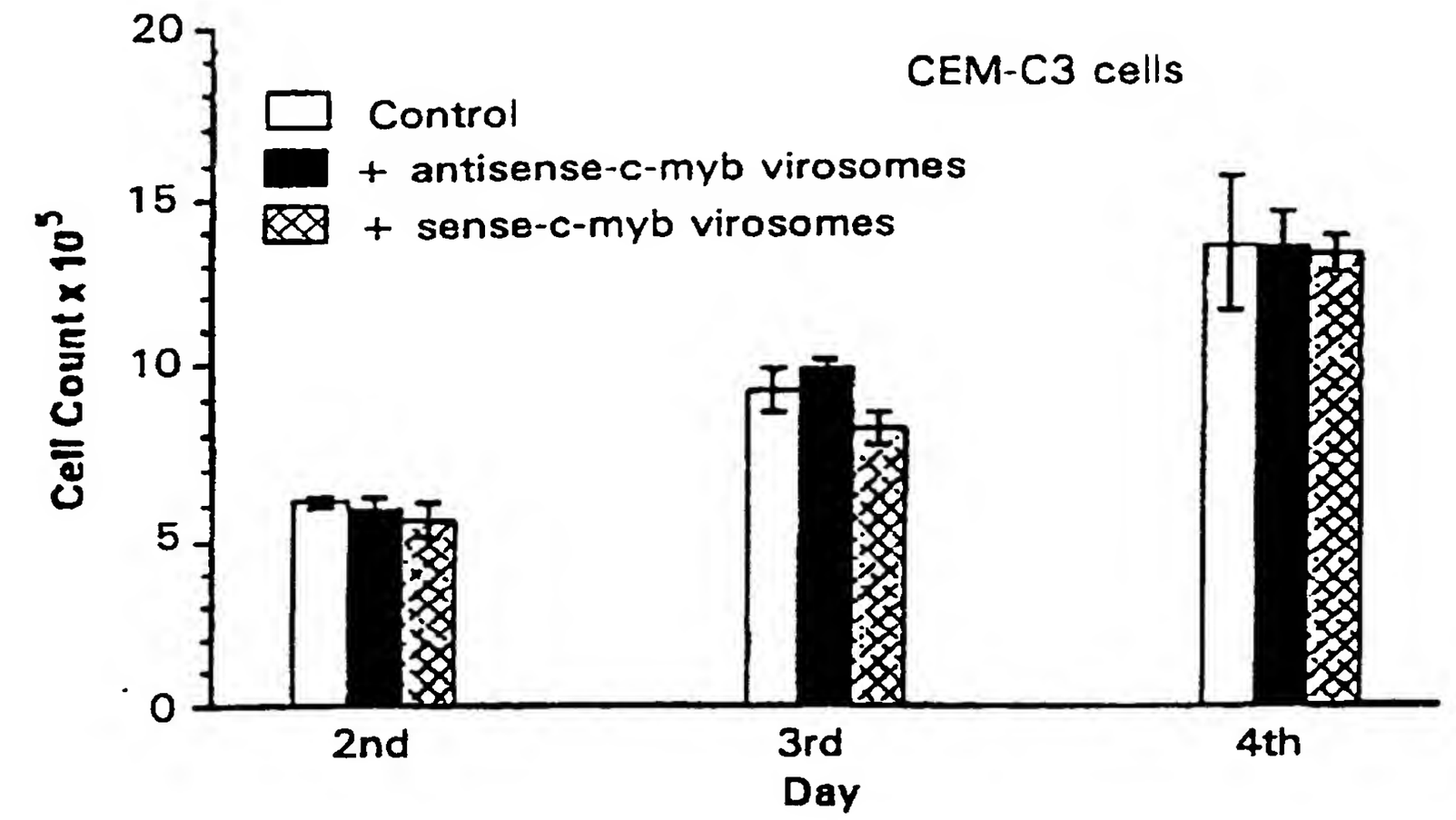

CEM-C3 cells
Control
+ antisense-c-myb virosomes
+ sense-c-myb virosomes
Cell Count x 10⁵
20
15
10
5
0
2nd
3rd
4th
Day

FIG. 18

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 97/02268

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K9/127 C12N15/88

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 32706 A (INEX PHARMACEUTICALS CORP. ET AL.) 7 December 1995 see claims 1-9,11,13-18,28 see page 2, line 20 - line 35 see page 7, line 31 - line 32 see page 9, line 6 - page 10, line 12 see page 15, line 19 - line 26	1,2,4,7, 9,11,15, 17, 23-25, 27-29, 32-34
Y	---	3,5,6, 10,14, 20-22
Y	WO 91 16024 A (VICAL, INC,) 31 October 1991 see abstract; claims 1,22-24	3,10

	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 August 1997	19. 09. 97
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	**Authorized officer** Alvarez Alvarez, C

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Int ional Application No
PCT/EP 97/02268

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 92 19267 A (SCHWEIZ. SERUM- & IMPFINSTITUT BERN ET AL.) 12 November 1992 see abstract; example 2 ---	5,6,14, 20-22
X	WO 95 02698 A (LIFE TECHNOLOGIES, INC.) 26 January 1995 see abstract; claims 1-4,7-14 see page 6, line 30 - page 7, line 27 see page 8, line 5 - line 14 see page 9, line 19 - line 26 see page 26, line 25 - page 27, line 27 see example 7 ---	1-4,7, 27,34
A	EP 0 497 997 A (NIKA HEALTH PRODUCTS LIMITED) 12 August 1992 see claims 1-6,12,14 & WO 92 13525 A cited in the application -----	

1

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

In tional Application No
PCT/EP 97/02268

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